



CMFRI SPECIAL PUBLICATION

Number 9

MANUAL OF RESEARCH METHODS FOR MARINE INVERTEBRATE REPRODUCTION



Issued on the occasion of the Workshop on
MARINE INVERTEBRATE REPRODUCTION
jointly organised by
the Department of Zoology, University of Madras and
the Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute, Cochin
held at the University of Madras
from 25th October to 10th November 1982

The Centre of Advanced Studies in Mariculture was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate agricultural education and research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to :

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Front cover : SEM picture showing surface topography of *Streptocephalus dichotomus* egg.

Manual of Research Methods for Marine Invertebrate Reproduction

EDITED BY

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OF MADRAS FROM 25TH OCTOBER TO 10TH NOVEMBER, 1982.

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PREFACE

The technologies of controlled reproduction, induction of spawning, sex reversal, artificial fertilisation, sterilisation and preservation of gametes are increasingly applied in aquaculture to obtain quality seed, quality fish stock and better yield. In this context, researches on different aspects of reproduction, developmental biology and physiology have assumed considerable importance besides their values in understanding of the ontogeny of the organisms. Extensive researches carried out in recent years from several laboratories in the world have not only accumulated a body of information, but also brought forth several new concepts to our understanding of the development and reproductive behaviour of finfishes and shellfishes.

In India, directed research on reproductive physiology and biology is taken up only recently and the field is still in an infant stage. In view of its emerging importance, it is identified as an area for priority research and for expertise development in the programmes of the Centre of Advanced Studies in Mariculture at the Central Marine Fisheries Research Institute, and several programmes of research are being taken up in this field with particular reference to the reproductive behaviour of the cultivable finfishes and shellfishes.

Advances made on the frontiers of invertebrate reproduction in recent years have been significant enough to organise a national workshop and to prepare a manual on research methodologies for the study of the subject. Several histological, histochemical and biochemical methods and sophisticated instruments have been introduced in these studies making it essential that the scholars who desire to work and specialise in the field are given adequate basic information on the research methods so as to enable them to appreciate and advance research to understand the problems confronted in the field.

The present manual, the third in the series, is prepared and compiled by Dr. T. Subramoniam, Leader of the 'Unit of

Invertebrate Reproduction ' of the Zoology Department of the University of Madras, Tamil Nadu. During the past decade, a team of research scholars are working on different aspects of marine invertebrate reproduction including the cultivable crustaceans such as *Scylla serrata*, *Panulirus homarus* and *Macrobrachium* spp. under his leadership. Contributing to our knowledge on the subject, the research results achieved so far in these aspects by the Unit have unfolded several new concepts in oogenesis, spermatogenesis, sperm transfer strategy, fertilization and endocrine control of reproduction and gamete formation.

I wish to express my great appreciation to Dr. T. Subramoniam and his team of Scholars, who by their dedication and interest evolved a series of tested research methods and set a theme of investigation through insight and skill on marine invertebrate reproduction. I am sure that this manual will be of immense use to the research scholars and scientists who would like to specialise in the subject and cognate fields.

This is the second workshop we are organising in close collaboration with the University of Madras. I wish to express my gratitude to Dr. M. Santappa, Vice-Chancellor, University of Madras for the keen interest evinced in such collaborative programmes and for the advice. I am also indebted to Dr. K. Ramalingam, Professor and Head of the Department of Zoology, University of Madras for productive discussions, continuous support and suggestions. I wish to thank Shri P. T. Meenakshisundaram and Shri K. Rengarajan, Scientists of the Central Marine Fisheries Research Institute for their help in the preparation of this manual.

E. G. SILAS,
Director, C.M.F.R.I.

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MARINE INVERTEBRATE REPRODUCTION: AN EXPERIMENTAL APPROACH*

Reproductive biology is central to biological science and an understanding of it is vital to proper animal management. Since marine invertebrates have representations in all phyla, some of them exclusively, their reproductive biology has been studied with the aim of extending the basic ideas over a wider phyletic and environmental range. Experimental studies on invertebrate reproduction in the past were limited to insects alone inasmuch as they formed the major agricultural pests. A proper control of them, however, entails a thorough knowledge of the sexual, reproductive and developmental biology.

With the advent of intensive aquaculture of useful marine invertebrates such as prawns, crabs and molluscs, not only a basic knowledge of the reproductive process of these invertebrates but an experimental approach to problems such as extrinsic and intrinsic factors controlling reproduction is very much in need. Basic information relating to reproductive periodicity, fecundity and mode of fertilization in the unstudied candidate species of marine invertebrates is, however, helpful in identifying the potential species for the purposes of aquaculture.

* Prepared by T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

The main area in which the experimental studies have been concentrated is the endocrine manipulation of reproductive activities such as gonad maturation and spawning. Again, gametogenesis, the central event in the reproductive cycle, is shown to be controlled by endocrine processes that differ markedly among the various invertebrate groups (Highnam, 1978). Perhaps, crustaceans have received the maximum attention in this regard in view of their aquaculture importance as well as the easy way in which the endocrinological manipulation can be achieved. For example, the localization of gonad inhibitory hormone in the eyestalk of decapod Crustacea is helpful in the easy extirpation of this source by simply ablating the eyestalk without much injury to the organism (Panouse, 1943). Similarly, implantation of neurosecretory organs such as brain and thoracic ganglia as well as grafting of ovary and androgenic glands have also been successful. In fact, most of our information on the endocrine regulation of reproduction in Crustacea has been obtained by such experimental studies (Subramoniam, 1981). A recent trend in the study of invertebrate endocrinology is organ culture, allowing the direct action of hormones on target tissues without interference from other systems (Gomot et al., 1980).

Among the marine invertebrates, the reproductive adaptations are in accordance with the life style of the organisms concerned. It is therefore difficult to generalize on the research methodologies for different groups of marine invertebrates. Bearing this in mind, the research methods given in this manual

have been so designed as to place the emphasis on the accurate assessment of morphological, structural and biochemical parameters used in the study of reproductive biology of typical marine invertebrate forms. The possibility of extending these methods to other invertebrates, showing variations in the reproductive anatomy and physiology is also explored.

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I. OOGENESIS AND ORGANIZATION OF THE OVARY

A HISTOLOGICAL CLASSIFICATION OF THE DEVELOPMENTAL STAGES OF CRUSTACEAN OOCYTE*

1.1. INTRODUCTION

Oogenesis is a dynamic process comprising i) a generative (proliferative) and ii) a vegetative (growth) phase. The generative phase refers to the mitotic multiplication of the primary oogonial cell (=gonocytes) into the secondary oogonial cell that transforms to primary oocyte. These events normally occur in the germinal zone (=germarium) of the ovary. The primary oocyte with a diploid number of chromosomes enters into the prophase of meiotic division. However, the meiotic divisions are arrested at the pachytene stage and the ooplasm starts accumulating yolk materials. This process is referred to as the vegetative phase and is normally completed in the growth zone (=vitellarium) of the ovary. The remaining stages of meiotic divisions are then quickly completed before or after the ovulation.

The morphological and functional characteristics of the oogonium and oocyte are given below :

OOGONIA :

The nucleus is very prominent and basophilic. Nucleolus, not distinguishable. The cytoplasm is in the form of a thin rim and lacks stainable material. The primary and secondary oogonial cells are not distinguishable under light microscope.

PRIMARY OOCYTES :

Previtellogenesis : Nucleus is transformed into a germinal vesicle. A prominent basophilic nucleolus is evident. Baso-

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philic granules (nucleolar extrusion bodies) are seen inside the nucleus and in the perinuclear region. Yolk materials are not detectable.

Vitellogenesis : Represents the period of rapid accumulation of yolk materials. The yolk is composed of yolk granules and yolk globules.

In general the decapod crustacean ovary undergoes changes in its coloration during maturation. This is due to the presence of carotenoid pigments linked to the main yolk protein. Therefore, the intensification of color is an index of the accumulation of the yolk protein. Based on color change as well as external morphology, the ovary is divisible into several stages. However, a corresponding histological examination of all stages should be made before finalizing the ovarian stages. The classification of the growing oocytes into the previtellogenic and vitellogenic stages are rather arbitrary as there is often overlapping of these two processes. Therefore this experiment is designed to make a correct assessment of the various ovarian stages both by external morphology and direct histological observations using an ocypod crab *Ocypoda platytarsis* (Milne Edwards).

1.2. MATERIAL

Ocypod crab *Ocypoda platytarsis* in different stages of ovarian maturation.

1.3. PROCEDURE

1.3.1. Morphological observations on the ovary

Take the female ocypod crab, remove the carapace, pick out the 'H' shaped ovary and find out the stages based on the criteria given below :

Immature

Stage I : Ovary colorless, thin and flimsy. Restricted only to cephalothoracic region. The ovary is hidden in the hepatopancreatic tissue.

Vitellogenesis-I :

Stage II : Ovary light yellow, transparent, posterior arms slightly extend to abdomen and are unequal.

Stage III : Ovary yellowish orange and flexible. The anterior arms extend and end near the gill chamber.

Stage IV : Ovary light orange, translucent, bulged and covered by transparent connective tissue layer.

Vitellogenesis-II

Stage V : Ovary orange and lobulated, opaque in nature, occupies the entire haemocoel.

Stage VI : Ovary deep orange, lobulated, oocytes are not very compact.

Stage VII : Ovary spent and colorless, flaccid, larger than immature and the arms extend upto abdomen.

1.3.2. Preparation of the paraffin sections of ovary

Fixation : Fixation helps preserving the structural integrity of intact animal, cells or tissue. Bouin's fixative (Saturated aqueous picric acid 75 ml; Formalin 25 ml; Glacial acetic acid 5 ml) is mainly used for early vitellogenic ovaries, whereas Ciaccio's fluid (Formalin 20 ml. ; 5% aqueous potassium dichromate 80 ml. and glacial acetic acid 5 ml) is recommended for late vitellogenic ovaries in which lipid yolk is enormous (Chou, 1957).

1. Fix the early vitellogenic ovaries in Bouin's fixative and the late vitellogenic ovaries in Ciaccio's fluid.
2. After 24 hours of fixation, wash the ovarian tissues repeatedly in running tap water until the yellow color of the Bouin's fluid is removed.
3. After washing the vitellogenic ovaries fixed in Ciaccio's fixative, soak the material in 3% potassium dichromate for 24 hours at room temperature and transfer the same to a saturated potassium dichromate solution and incubate at 37°C for one week.

4. The ovary is then dehydrated in a series of alcohol from 30% to 100%.
5. Clear the ovary either in xylene or methyl salicylate. Take care to avoid the material becoming brittle.
6. Transfer the transparent ovary into the molten wax (melting point 52-54°C) already kept in the oven.
7. After complete infiltration, make blocks of the ovary in fresh molten wax.
8. Cut sections at 6-8 μ m in a rotary microtome.
9. Take a clean dry slide and apply a drop of Mayer's glycerol albumen adhesive, a combination of fresh egg white and glycerol (1 : 1).
10. Spread the sections over the slide with the help of hot plate.
11. Dewax the sections in xylene and hydrate the slides in series of alcohol from 100% to 30% and then in distilled water.
12. Stain the slides in Ehrlich's haematoxylin and counter stain in 1% aqueous eosin (Bancroft and Stevens, 1977).
13. Dehydrate the slides through alcohol series and mount in DPX.

1.4. OBSERVATION

1. Observe the placement of germinal zone in the immature ovary. Distinguish the oogonial and follicle cells in the germinal zone based on the shape and tinctorial properties of the cells.
2. Observe the behaviour of follicle cells in different stages of vitellogenesis.
3. Observe the changes in the nuclear morphology and the ooplasmic content.
4. Observe the stages in the oosorption of relict oocytes and recuperation of ovary after ovulation.

1.5. MICROMETRIC MEASUREMENT OF OOCYTES IN DIFFERENT STAGES OF OVARY

Size increase of the oocyte is a function of oogenesis, and hence micrometric measurements of oocytes in different stages of maturation will provide an important criteria for classifying the oocytes.

1.5.1. Procedure

1. Since oocytes deviate strongly from a spherical shape, measure the longest and shortest axes of oocyte diameter using ocular micrometer (Gonor, 1973).
2. Calculate the area of oocytes using the formula πr^2
3. Plot the values of oocyte area against percentage of oocytes in the ovarian stages classified as above, in the form of histogram (Laulier and Demeusy, 1974).

1.5.2. Inference

The histogram (Fig.1) represents data on the gradual size increase of oocyte during ovarian maturation in the ocypod, *Ocypoda platytarsis*. The presence of different size classes in each ovarian stage suggests that there is intermittant spawning of eggs within a particular breeding season.

1.6. OBSERVATION ON OOCYTE MATURATION OF THE OCYPOD CRAB USING CRYOCUT SECTIONS OF UNFIXED OVARY

The cryocut was originally devised by Linderstrom-Lang and Mogenson (1938) for quantitative cytochemical investigations. In this technique unfixed frozen tissues are used for enzyme and lipid histochemistry. For immediate observation of cytological details in the ovary, cryocut sections are employed in the present experiment.

The cryostat is a well insulated chamber equipped with a rotary microtome. The temperature of the chamber is automatically maintained at a low temperature, between -5°C and -30°C .

TABLE 1. *Area and the percentage of different stages of oocytes (OC; PVO; VO) in different ovarian stages of Ocypoda platytarsis*

Stage with colour of the ovary	Oogonial cells		Previtellogenic oocytes		Vitellogenic oocytes	
	Area in mm ²	Percentage	Area in mm ²	Percentage	Area in mm ²	Percentage
Light yellow (St. II)	.. 0.265	57.00	1.658	30.00	4.78	13.00
Yellowish orange (St. III)	.. —	—	2.030	70.59	5.95	29.41
Light orange (St. IV)	.. —	—	1.714	36.36	9.355	63.64
Orange (St. V)	.. —	—	1.170	22.58	12.234	77.42
Deep orange (St. VI)	.. —	—	1.145	13.30	24.185	86.70

OC — Oogonial cells
PVO — Previtellogenic oocytes
V — Vitellogenic oocytes.

$$\text{Area in mm}^2 = \pi r^2; \quad r = \frac{r_1 + r_2}{2}$$

r_1 — radius of long axis of oocyte

r_2 — radius of short axis of oocyte

$$\text{Percentage of cells} = \frac{\text{Number of individual stage oocytes}}{\text{Total number of different stages of oocytes}} \times 100.$$

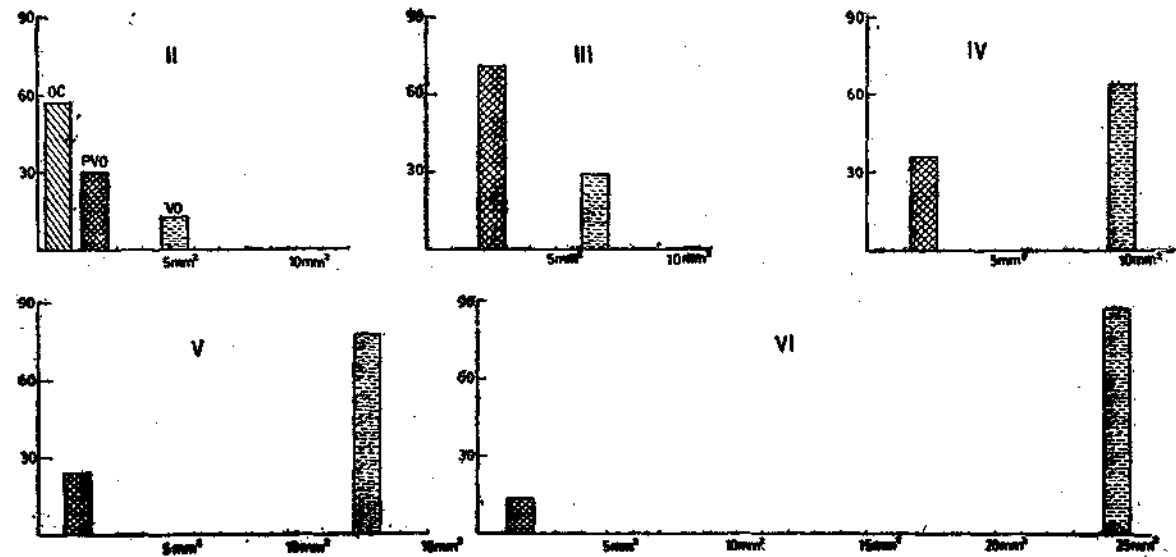


Fig. 1

Growth sequence of female gametes during gametogenic cycle of *Ocypoda platytarsis*.

Abscissa—Area of stages of oocytes in mm²; Ordinate—Percentage of different stages of oocytes in ovarian stages; II-IV—Substage a, b and c of vitellogenesis I; V-VI—Substage a and b of vitellogenesis-II.

1.6.1. Procedure

Block making

1. Adjust the temperature of the cabinet to -20°C .
2. Use 40% sucrose or distilled water as freezing agent for fixing the material on the tissue holder or object disc.
3. Excise a small bit of fresh ovary and place in the tissue holder and add 40% sucrose solution drop by drop.
4. Place the tissue holder in the heat sink, which by possessing high thermal conductivity, draws the heat from the ovarian tissue. By this process, the tissue remains in a frozen condition.

Section cutting

1. Use 120 mm microtome knife and fix in correct angle.
2. To rapidly advance the ovary towards the knife, use the crank on the fast feed knob in the clockwise direction.
3. For fine adjustments of ovary in position, use the knurled knob portion of the control.
4. Adjust the black set screw knob on the backside of the thickness scale to cut sections at 8 to 10 μm .
5. Take sections by rotating the hand which yield single separate sections. Collect the sections in an embryo cup or transfer directly to glass slide using fine camel hair brush.
6. Stain in haematoxylin and eosin and observe the cytological details of all stages of ovary under microscope.

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II. VITELLOGENESIS

HISTOCHEMICAL PROCEDURES FOR CHARACTERIZING THE CRUSTACEAN YOLK*

2.1. INTRODUCTION

Histochemical studies on yolk formation in crustaceans not only reveal the chemical nature of various yolk substances but also provide information on the temporal pattern of yolk accumulation and their spatial distribution. In crustaceans the accumulation of yolk material starts with the dispersion of nucleolar extrusions in the ooplasm. The yolk protein to be detected first in the ooplasm is in general glycolipoprotein in nature. This is followed by the accumulation of a glycolipoprotein substance that is presumed to be originating from extra-ovarian sources. Apart from this, discrete lipid globules have also been found to be deposited at various stages of vitellogenesis. In any study on vitellogenesis, a preliminary histochemical characterization of the sequentially deposited yolk materials is essential in view of the variability in the biochemical composition of yolk among different crustacean species. Such differences in the biochemical composition of yolk in turn reflects the nature of embryonic development. In the present experiment a battery of histochemical procedures is given to detect the major deutoplasmic substances such as the basophilic granules, (ribosomal RNA), protein, carbohydrate and lipid. The rationale of the tests is also given along with the procedure.

2.2. MATERIALS

Paraffin and cryocut sections of the ovary of the anomuran crab, *Emerita asiatica*.

* Prepared and verified by Sudha Varadarajan, Zoological Survey of India, Madras, M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

2.3. PREPARATION OF FIXATIVES

1. *Neutral buffered formaldehyde* : Add 100 ml 40% formaldehyde to 900 ml distilled water. To the above solution, dissolve 4 gm sodium dihydrogenphosphate and 6.5 gm disodium hydrogenphosphate. Shake well.
2. *Carnoy's* : Add 60 ml ethyl alcohol to 30 ml chloroform. To this, add 10 ml glacial acetic acid.
3. *Formol-calcium* : Add 100 ml 40% formaldehyde to 500 ml distilled water. Dissolve 10 gm anhydrous granular calcium chloride to the above solution. Shake well and make upto 1 litre. Store with marble chips.

2.4. TESTS FOR PROTEINS

2.4.1. Mercuric Bromophenol Blue Test for Protein

Principle

Mercuric ions of the bromophenol blue solution react with acidic, sulphhydryl and aromatic residues of the protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

Mercuric bromophenol blue : Dissolve 1 gm mercuric chloride and 0.05 gm bromophenol blue in 100 ml 2% aqueous acetic acid.

Method

Bring sections to water. Stain in the mercuric bromophenol blue solution for 2 hours at room temperature. Rinse sections for 5 minutes in 0.5% acetic acid. Transfer sections directly into tertiary butyl alcohol. Clear in xylene and mount in DPX.

Result

Proteins—deep blue colour.

2.4.2. Aqueous Bromophenol Blue Test for Basic Proteins

Principle

Bromophenol blue is an acidic dye which in aqueous medium is capable of reacting with the basic reactive groups. The acidic groups of the dye react with basic groups of the protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

0.1% *Bromophenol blue* : Dissolve 0.1 gm bromophenol blue in 100 ml double distilled water.

Method

Bring sections to water and stain in 0.1% bromophenol blue solution for 5 minutes at room temperature. Wash in double distilled water and observe.

Control : Deamination (*vide* section 2.7.1.).

Result

Basic proteins—blue.

2.4.3. Ninhydrin-Schiff Test for Amino Groups

Principle

In the course of oxidative deamination with ninhydrin stable tissue aldehydes are produced. These are demonstrated with Schiff's reagent.

Fixation and Section

Carnoy's ; paraffin, cryostat.

Reagents

1. 0.5% *Ninhydrin* : Dissolve 0.5 gm ninhydrin in 100 ml absolute alcohol.
2. *Schiff's reagent* : Dissolve 1 gm basic fuchsin in 200 ml boiling distilled water. Shake for 5 minutes and cool to

keep in exactly 50°C and add to the filtrate 20 ml 1 N hydrochloric acid ; cool to 25°C and add 1 gm sodium metabisulphite. Stand in the dark for 14-24 hours in frig. Add 2 gm activated charcoal and shake for 1 minute. Keep the filtrate in the dark at 0-4°C. Allow to reach room temperature before use.

Method

Bring sections to water. Treat sections with 0.5% ninhydrin solution for 16-20 hours at 37°C. Wash gently in running water, 2-5 minutes. Immerse in Schiff's reagent, 15-25 minutes. Wash in running tap water, 10 minutes. Dehydrate, clear and mount in DPX.

Control : Deamination (*vide* section 2.7.1.).

Result

Amino groups—pinkish red to magenta.

2.4.4. Toluidine Blue Test for Acidic Groups

Principle

Toluidine blue is a basic dye which in aqueous medium reacts with the acidic groups of protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

1% *Toluidine blue* : Dissolve 1 gm toluidine blue in 100 ml of double distilled water.

Method

Bring sections to water. Stain in 1% toluidine blue for 10 minutes. Wash in water and observe.

Control : Methylation (*vide* section 2.7.2.)

Result

Acidic group—red or pink or purple ; Nuclei—blue.

2.4.5. Ferric-Ferriyanide Method for—SH Groups

Principle

This method depends on the reduction of a fresh solution of potassium ferriyanide in acid solution at pH 2.4 by sulphhydryl groups in the tissues. The resulting ferriyanide combines with ferric ion (in ferric sulphate) to give an insoluble prussian blue precipitate.

Fixation and Section

10% neutral buffered formalin ; paraffin, cryostat.

Reagent

Ferriyanide reagent : Add 3 parts of 1% aqueous ferric sulphate to 1 part of 0.1% aqueous potassium ferriyanide and adjust to pH 2.4.

Method

Wash sections in distilled water. Immerse in 3 changes of the ferric cyanide reagent for 20-25 minutes (paraffin sections) or 10-20 minutes (fresh smears). Wash in distilled water. Dehydrate, clear and mount. Brief rinsing in 2% alkaline alcohol (2 gm NaOH in 60% alcohol) before dehydration reduces diffuse blue background staining.

Control : Mercaptide (*vide* section 2.7.4.)

Result

Sulphydryl groups—blue.

2.4.6. Thioglycollate Ferric-Ferriyanide Method for SS Groups

Principle

Unreactive disulphide groups are reduced to reactive sulphydryl groups by thioglycollate. The sulphydryl groups reduce the ferriyanide. The other reactions are as given in 2.4.5.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

2.5% Sodium thioglycollate : Dissolve 2.5 gm sodium thioglycollate in 100 ml double distilled water and adjust to pH 8.

Method

Treat 2 sets of sections simultaneously. Bring both sections to water, then immerse them for 30 minutes in 2.5% sodium thioglycollate. Wash in weakly acidified distilled water (pH 4) for 3 minutes ; wash in running tap water for 3 minutes. Rinse in distilled water. Transfer the 2nd (control) slide to phenyl mercuric chloride in butanol (48 hours). After blocking, bring back to water. Both sections should now be immersed in a fresh solution containing 10 ml freshly made 1% aqueous potassium ferrocyanide and 30 ml 1% aqueous ferric chloride (just filtered). Leave for 1 minute. Wash in 3 changes of distilled water for 10 minutes. Dehydrate, clear and mount in DPX.

Control : Thioglycollate reduction (*vide* section 2.7.7.).

Result

Disulphide groups—prussian blue.

2.4.7. Millon's Test for Tyrosine

Principle

When Millon's reagent, a mixture of mercurous and mercuric nitrates and excess of nitric acid, is added to the protein and the mixture is heated for few minutes, a white precipitate is formed which may turn yellow and then red if the reacting protein contains tyrosine. The reaction is specific for hydroxy phenyl groups unsubstituted in the meta position.

Fixation and Section

10% neutral buffered formalin ; paraffin or cryostat.

Reagent

Millon's reagent : Add 10 gm mercuric sulphate to 100 ml 10% sulphuric acid and heat until dissolved. Make up to 200 ml. Add 0.5 ml. 0.25 % aqueous sodium nitrite solution to 5 ml of the above solution.

Method

Hydrate sections, place them in a small beaker containing the reagent and leave in an oven until low boil. Bring sections to room temperature. Remove sections and wash in distilled water, three times (wash each slide for 2 minutes). Dehydrate, clear and mount in DPX. Repeat with fresh tissue smears and note the difference.

Control : Iodination (*vide* section 2.7.5.).

Result

Tyrosyl groups—red or pink.

2.4.8. DMAB-Nitrite Method for Tryptophan

Principle

The aldehyde component of the p-dimethylamino-benzaldehyde (DMAB) solution reacts with the tryptophanyl reactive sites and forms a blue coloured compound called β -carboline pigment. Sodium nitrite solution is used to intensify the colour of the β -carboline pigment.

Fixation and Section

Fresh material ; cryostat.

Reagent

5% DMAB : Dissolve 5 gm DMAB in 100 ml hydrochloric acid.

Method

Bring sections to absolute alcohol and allow them to become just dry in the air at room temperature. Immerse sections in 5% DMAB for 1 minute. Transfer to 1% sodium nitrite solution in concentrated hydrochloric acid for a further minute. Wash in tap water for 30 seconds. Rinse in 1% acid alcohol, dehydrate, clear and mount in DPX.

Control : Formaldehyde (*vide* section 2.7.6.).

Result

Tryptophanyl groups—deep blue.

2.5. TESTS FOR CARBOHYDRATES

2.5.1. Periodic Acid—Schiff Technique

Principle

Periodic acid, an oxidant breaks the C-C bonds where these are available as 1,2 glycols, converts them into dialdehydes but does not oxidise the aldehyde further and these can be localised by Schiff's reagent.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. *Periodic acid* : Dissolve 0.4 gm periodic acid in 35 ml ethyl alcohol. Add 5 ml 0.2 M Sodium acetate (27.2 gm of the hydrated salt in 1000 ml) to 10 ml distilled water. Keep in dark at 17°C—22°C and use at this temperature. Discard if brown colour appears.
2. *Schiff's reagent* : (vide section 2.4.3.).

Method

Bring sections to water. Oxidise for 10 minutes in periodic acid. Wash in running water : 5 minutes. Immerse in Schiff's reagent : 10 minutes. Wash in running water : 5 minutes. Dehydrate and mount in DPX.

Control : Deamination, acetylation, deacetylation, chloroform/ methanol extraction, pyridine extraction and taka diastase (vide sections 2.7.1., 2.7.8—2.7.12.).

Result

Glycogen—deep magenta ; Other hexose containing muco substances—shades of purplish red.

2.5.2. Best's Carmine Test for Glycogen

Principle

Carminic acid at a pH on the alkaline side of its isoelectric point is negatively charged and behaves like an acid dye staining 1, 2 glycol groups, perhaps by hydrogen bonding.

Fixation and Section

Carnoy's ; paraffin.

Reagents

1. *Carmines stock solution* : Add 2 gm carmine, 1 gm potassium carbonate and 5 gm potassium chloride to 60 ml distilled water. Boil gently for 5 minutes, cool and filter. Add 20 ml ammonia (Sp. gr. 0.88) to the filtrate. This solution lasts for 3 months at 0°—4°C.
2. *Carmines staining solution* : Dilute 15 ml stock solution with 12.5 ml ammonia (Sp. gr. 0.88) and 12.5 ml methyl alcohol. This solution lasts for 2-3 hours.
3. *Best's differentiator* : To 8 ml absolute alcohol, add 4 ml methyl alcohol and 10 ml distilled water.

Method

Bring sections to absolute alcohol. Place sections in 1% celloidin in absolute alcohol/ether (equal parts) for 2 minutes. Dry in air. Down grade to water. Stain in Ehrlich's haemalum : 5 minutes. Rinse and differentiate rapidly in 1% acid alcohol. Rinse in water. Stain in Best's carmine, 15-30 minutes. Differentiate in Best's differentiator without rinsing (5-60 seconds). Wash in 80% alcohol. Dehydrate in absolute alcohol. Clear in xylene and mount in DPX.

Control : Taka diastase (*vide* section 2.7.12.).

Result

Glycogen : red ; Nuclei : dark blue.

2.5.3. Toluidine Blue at different pH for Acid Mucopolysaccharides

Principle

Toluidine blue, a basic dye reacts with acid mucopolysaccharides (AMP) at different pH. At lower pH the dye colours the sulphated AMP whereas in higher pH it stains the phosphated

AMP. The metachromasia at lower and higher pH indicates the presence of sulphated and carboxylated AMP respectively.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. **Solution A :** Dissolve 40 mg toluidine blue in 25 ml 1 N sodium acetate solution (8.25 mg sodium acetate in 100 ml distilled water)
2. **Solution B :** 1 N hydrochloric acid. (Add 90 ml of the acid to 910 ml distilled water). Prepare toluidine blue at different pH as given below.

pH	Solution A	Solution B
1.09	20 ml	28 ml
1.99	20 ml	21 ml
3.09	20 ml	19.4 ml
4.19	20 ml	15 ml

7.00. Dissolve 8 mg toluidine blue in 20 ml distilled water.

Method

Bring sections to water and stain in toluidine blue at different pH for 20 minutes, wash in distilled water and observe.

Control : Methylation (*vide* section 2.7.2.).

Result

AMP	—blue
pH 1.09	—sulphated AMP
pH 1.99	—sulphated or phosphated AMP
pH 3.09 }	—sulphated AMP
pH 4.19 }	
pH 7.00	—carboxylated AMP

2.5.4. Critical Electrolyte Concentration (CEC) Method for Acid Mucopolysaccharides

Principle

Both sulphated mucins and glucosaminoglucouranoglycans containing carboxyl groups will bind with alcian blue *in situ* in

the presence of low concentrations (below 0.3 M) of electrolytes whereas only sulphated mucosubstances will do so with higher concentration (above 0.8 M).

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1% Alcian blue : Dissolve 1 gm alcian blue 8GX in 100ml 0.05 M sodium acetate buffer (410 mg sodium acetate in 100 ml distilled water) at pH 5.7.

Prepare different molar concentrations of alcian blue using the table given below :

1% Alcian blue		Magnesium chloride		Molarity
25 ml	+	0.508 gm	—	0.1 M
25 ml	+	1.016 gm	—	0.2 M
25 ml	+	2.540 gm	—	0.5 M
25 ml	+	3.048 gm	—	0.6 M
25 ml	+	4.064 gm	—	0.8 M
25 ml	+	5.080 gm	—	1.0 M

Method

Bring sections to water. Stain in alcian blue for 30 minutes at different CEC. Wash in running water for 5 minutes. Dehydrate quickly in alcohols, clear in xylene and mount in DPX.

Result

Hyaluronic acid, sialomucins and some weakly acidic sulpho-mucins are not stained at or above 0.1 M magnesium chloride. Most sulphated mucosubstances stain strongly at 0.2 M levels. The various sulphated mucosubstances lose alcianophilia at different levels with increasing magnesium chloride concentration.

2.5.5. Bracco-Curti's Test for Sulphated Acid Mucopolysaccharides

Principle

Benzidine in 2% boric acid reacts with the sulphate groups of AMP to form benzidine sulphate. Potassium dichromate

oxidises the benzidine sulphate to give benzidine blue colour indicating the presence of sulphated acid mucopolysaccharides.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. 1% *Benzidine* : Dissolve 1 gm benzidine in saturated aqueous solution of boric acid.
2. 2% *Boric acid* : Dissolve 2 gm boric acid in 100 ml distilled water.
3. 1% *Potassium dichromate* : Dissolve 1 gm potassium dichromate in 100 ml distilled water.

Method

Bring sections to water and treat in benzidine boric acid mixture for 10 minutes. Wash with 2% boric acid thrice. Treat in 1% potassium dichromate solution for 30 minutes. Wash in distilled water and observe.

Result

Sulphated AMP—benzidine blue.

2.6. TESTS FOR LIPIDS

Principle of lipid staining techniques in general

Lipid histochemistry is dependent on the solubility of the dyes in the fat themselves. The commonest dyes used for this purpose are Sudan black B, Oil red 'O' and Nile blue. Staining with these dyes depends largely on the type and concentration of the fluid in which they are dissolved or suspended ; but it is imperative that the solubility of the dye in fat exceeds its solubility in the solvent. Coupled with these techniques, extraction procedures are almost always employed where, after application of known lipid solvents, lipid tests are applied and the results compared with unextracted material.

2.6.1. Sudan Black B Test for Lipid

Principle

This is a diazo dye, and being slightly basic because of its amino groups, combines with the acidic groups of compound lipids such as phospholipids.

Fixation and Section

Formol-calcium ; frozen.

Reagents

1. *Sudan black B* : Prepare saturated solution of Sudan black B in 70% ethanol. Keep aside for a week.
2. *Differentiator* : 70% alcohol.

Method

Stain in Sudan black B for 15 minutes ; differentiate in 70% alcohol until a delipidized control section appears colourless, dry and mount in glycerine jelly. Treat pyridine extracted materials in the same manner.

Control : Pyridine extraction/Chloroform: methanol extraction (*vide* sections 2.7.10 ; 2.7.11.).

Result

Bound lipids and lipids—black or dark blue.

2.6.2. Nile Blue Method for Neutral and Acidic Lipids

Principle

Neutral lipids will dissolve out of aqueous solutions of Nile blue, only the oxazone and the free base (both red). Acidic lipids will dissolve the oxazone and combine with the free base to form blue lipid-soluble compounds.

Fixation and Section

Formol—calcium ; frozen.

Reagents :

1. *Sudan black B* : *vide* section 2.6.1.
2. 1% *Nile blue* : Dissolve 1 gm. Nile blue in 100 ml of distilled water.

3. 0.02% *Nile blue* : Dissolve 20 mg Nile blue in 100 ml distilled water.
4. 1% *Acetic acid*: Add 1 ml acetic acid to 99 ml distilled water.

Method

Stain one section (A) in Sudan Black B in 70% alcohol as a control for lipid. Stain section B in 1% Nile blue at 60°C for 5 minutes. Wash quickly in water at 60°C for 5 minutes and differentiate in 1% acetic acid at 60°C for 30 seconds.

Stain another section C as B and restain in 0.02% Nile blue at 60°C. Wash and differentiate the section (as section B). Mount all sections in glycerine jelly.

Pyridine extracted control sections are stained with Sudan black B and compared with unextracted one. If there is no difference between B and C, the first may be discarded as what will stain with 1% Nile blue will also stain in 0.02%.

Control : Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11.).

Results

Neutral lipids—red.

Acidic lipids—blue

2.6.3. Nile Blue Method for Phospholipids

Since Nile blue Principle stained phospholipids are weakly acid fast while similarly stained proteins are not, a two stage differentiation, first with acetone at 50°C followed by one with weak acid is introduced into the Nile blue method.

Fixation and Section

Formol—calcium ; cryostat.

Reagent

Nile blue sulphate : Mix 500 ml saturated aqueous Nile blue sulphate solution with 50 ml 0.5% aqueous sulphuric acid. Boil for 2 hours before use.

Method

Stain for 90 minutes at 60°C in Nile blue sulphate solution. Rinse in distilled water. Place in acetone heated to 50°C. Remove sections from source of heat but allow sections to remain in it for 30 minutes. Differentiate in 5% acetic acid for 30 minutes. Rinse in distilled water. Differentiate again in 0.5% aqueous hydrochloric acid for 3 minutes. Wash in distilled water and mount in glycerine jelly.

Control : Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11).

Result

Phospholipids—blue.

2.6.4 Oil Red 'O' Method for Neutral Lipids

Oil red 'O' is superior to the red Sudan dyes as the colour is deeper, smaller droplets are better seen and there is less tendency to the formation of dye precipitates.

Fixation and Section

10% neutral buffered formalin ; cryostat.

Reagents

1. *Stock solution* : Add 0.5 gm oil red 'O' to 100 ml 98% isopropanol.
2. *Staining solution* : Dilute 6 ml of the stock solution with 4 ml of water, stand for 24 hours and filter. Use this as a stock staining solution, filtering through Whatman No. 42 paper, sufficient amounts as and when necessary.

Method

Stain frozen sections after rinsing in water and then in 60% isopropanol, in freshly filtered oil red 'O' solution for 10 minutes. Differentiate briefly in 60% isopropanol. (Keep tightly stoppered or make up fresh). Wash in running water for at least 10 minutes. Mount in glycerine jelly.

Control : Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11).

Result

Neutral lipids—red.

2.6.5. U. V. Schiff Reaction for Unsaturated Lipids

Principle

Fresh smears or frozen sections, if subjected to long and short wave (254 nm) U. V. irradiation for 3-4 hours, treated Schiff's reagent and compared with untreated intact controls, the difference in staining intensity demonstrated the number of double bonds saturated by oxidation.

Fixation and Section

Cold neutral buffered formalin; cryostat.

Reagent

Schiff's reagent: *vide* section 2.4.3.

Method

Fix sections for 12-18 hours in cold 10% neutral buffered formalin. Place under a source of ultraviolet light for 2-4 hours. Treat with Schiff's reagent : 15 minutes. Rinse with 3 changes of sulphurous acid water. Rinse in distilled water. Mount in glycerine jelly.

Control : Pyridine extraction (*vide* section 2.7.11.).

Result

Magenta colour absent from unirradiated control sections indicates unsaturated lipids.

2.6.6. Sudan Black 'B' Method for Masked Lipids

Principle

Pretreatment of tissue with various organic acids (acetic, citric, oxalic) and subsequent staining with a ripened 70% alcoholic Sudan black B would demonstrate the lipids, unmasked by the pretreatment.

Fixation and Section

Formalin vapour ; cryostat.

Reagents

1. *Sudan black B* : vide section 2.6.1
2. *70% Alcohol* : Add 70 ml alcohol to 30 ml distilled water.

Method

Use fresh smears fixed in formalin vapour for 2-5 minutes. Immerse films in 25% aqueous acetic acid. Wash thoroughly in tap water, then distilled water and allow to dry. Stain in Sudan black B (this solution should be atleast one week old). Differentiate in 70% alcohol. Blot dry and mount in glycerine jelly.

Control : Pyridine extraction (vide section 2.7.11).

Result

Bound lipids and lipids—black.

2.7. BLOCKING PROCEDURES

The blocking procedures generally serve to prove the presence of the specific reactive group and the removal of interfering groups. Histochemical tests should therefore be carried out with suitable controls.

2.7.1. Deamination

Reagents

1. *3% Sulphuric acid* : Add 3 ml concentrated sulphuric acid to 97 ml distilled water.
2. *1% Sodium nitrite* : Dissolve 1 gm sodium nitrite in 100 ml 3% aqueous sulphuric acid.

Method

Immerse the hydrated sections in 1% sodium nitrite solution for 48 hours at 5°C.

2.7.2. Methylation

Reagent

1 N Hydrochloric acid : Measure 91.2 ml methanol and to it add 8.8 ml concentrated hydrochloric acid.

Method

Treat the hydrated sections with 1 N hydrochloric acid for 96 hours at 37°C.

2.7.3. Demethylation

Reagent

5% Potassium permanganate : Dissolve 5 gm potassium permanganate in 100 ml distilled water.

Method

Treat the methylated sections with 5% potassium permanganate for 20 minutes at 37°C.

2.7.4. Mercaptide

Reagent

Mercuric chloride : Prepare a saturated solution of Mercuric chloride in distilled water. Immerse the hydrated sections in the mercuric chloride solution for 1 hour at 30°C.

2.7.5. Iodination

Reagents

1. *Iodine solution* : Dissolve 1 gm iodine and 2 gm potassium iodide in 300 ml distilled water.
2. *3% Ammonia* : Add 3 ml ammonia with 97 ml distilled water.

Method

Treat the hydrated sections with iodination reagent for 24 hours at 30°C.

2.7.6. Formaldehyde

Treat the hydrated sections with 40% formaldehyde for 1 hour at 30°C.

2.7.7. Thioglycollate Reduction

Reagents

1. *0.5 M Thioglycollate* : Dissolve 4.6 ml thioglycollic acid in 100 ml distilled water (adjust to pH 8 with 0.1 N sodium hydroxide).
2. *0.1 N Sodium hydroxide* : Dissolve 400 mg sodium hydroxide in 100 ml distilled water.

Method

Treat the sections with 0.5 M thioglycollate for 4 hours at 37°C.

2.7.8. Acetylation

Reagent

Acetic anhydride and pyridine mixture : Mix equal volume of acetic anhydride and pyridine solutions.

Method

Immerse the hydrated sections in acetic anhydride and pyridine mixture for overnight at 22°C.

2.7.9. Deacetylation

Reagents

1. *70% Alcohol* : Add 30 ml distilled water with 70 ml ethyl alcohol.
2. *1% Potassium hydroxide* : 1 gm potassium hydroxide in 100 ml 70% alcohol.

Method

Treat the acetylated sections with 1% potassium hydroxide for 20 minutes at room temperature.

2.7.10. Chloroform Methanol Extraction

Keep the hydrated sections in chloroform: methanol (1 : 1) mixture for 18 hours at 60°C.

2.7.11. Pyridine Extraction

Reagents

1. *Bouin's fluid*: vide expt. No. 1.
2. *Dichromate—calcium*: Dissolve 5 gm potassium dichromate and 1 gm anhydrous calcium chloride in 25 ml distilled water and make it up to 100 ml.

Method

Fix in weak Bouin's fluid for 20 hours. Wash in alcohol. Immerse in pyridine at 17°—22°C for 30 minutes; immerse in pyridine at 60°C for 24 hours. Wash in running water for 2 hours. Transfer to dichromate—calcium mordant.

2.7.12. Taka Diastase Treatment

Treat the sections with taka diastase for 20 minutes at room temperature.

2.8. OBSERVATION

Tabulate the histochemical reactions for protein, carbohydrate and lipid obtained on the various yolk components of the oocytes in different stages of maturation. Also indicate the intensity of reaction.

2.9. REFERENCE

- PEARSE, A. G. E. 1968. *Histochemistry: Theoretical and applied*. Vol. I. 3rd Edn., J & A. Churchill Ltd., pp. 758.

**IDENTIFICATION AND CHARACTERIZATION
OF VITELLOGENIN AND LIPOVITELLIN
OF *SCYLLA SERRATA* AND *EMERITA ASIATICA*
USING DISC GEL ELECTROPHORESIS***

3.1. INTRODUCTION

The appearance of a sex limited plasmatic protein (FSP) in the mature crabs and other higher crustaceans is now well established. As early as 1954, Frentz observed the FSP in the blood of *Carcinus maenas* during vitellogenesis. This protein is considered to be the precursor of the main yolk protein of the egg. The occurrence of FSP in the blood is a secondary sexual feature inasmuch as they indicate the specific stage of vitellogenesis during gametogenic cycle. Earlier workers characterised the chemical nature of this protein to be a very high density lipoprotein in a few crustacean species (Wallace *et al.*, 1967; Fyffe and O' Connor, 1974). Identification of vitellogenin in the blood as well as the lipovitellin of the egg is important not only for studying the mode of yolk formation but also for identification of maturity stages in the females during the breeding season.

3.2. PRINCIPLE

The charged biological molecules depending on the pH and suspending medium migrate to the electrodes of opposite polarity in an electrical field. Polymerization of acrylamide and cross-linking reagent methylenebisacrylamide is done in the presence of a catalyst, ammonium persulphate. Tetramethylethylenediamine (TEMED) initiates and controls polymerization. The electrophoretic mobility of the glycinate ion is very much less. However in the pH of the

* Prepared and verified by S. Ezhilarasi and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

separation gel (8.9) the mobility of glycine is greater than that of the protein, and hence the buffer always runs ahead the protein molecules. Bromophenol blue, used as the marker dye, having a low molecular weight, marks the boundary for the protein molecules and runs ahead the protein. Vitellogenin and lipovitellin of crustaceans being high density glycolipoprotein complexes are well separated on the polyacrylamide gel.

Acrylamide disc gel electrophoresis has been carried out according to Davis (1964) using his original stock and buffer systems. For fractionating the proteins of haemolymph and ovary of *S. serrata* both spacer and sample gels were omitted, since good separations were obtained without spacer and sample gels (Clark, 1964). In *S. serrata*, the haemolymph resolved into one to eight slow moving fractions, three distinct medium moving fractions and three to four fast moving fractions. This pattern however changes with the stage of ovarian maturity. Subhashini and Ravindranath (1981) have reported that the resolution of fractions is better when they used spacer gel for separating the haemolymph proteins of *S. serrata*.

3.3 Reagents

1. *Stock monomer solution* : Dissolve 25 gm of acrylamide with 0.735 gm of N, N-methylenebisacrylamide in 100 ml of double distilled water.
2. *Small pore buffer* : Dissolve 36.6 gm of Tris with 0.23 ml of TEMED in 48 ml of 1N hydrochloric acid and make it up to 100 ml with double distilled water. To prepare 1N hydrochloric acid dilute 9 ml of hydrochloric acid to 100 ml with double distilled water.
3. *Catalyst* : Dissolve 0.14 gm of ammonium persulphate in 100 ml of double distilled water.
4. *Stock reservoir buffer (pH 8.3)* : Dissolve 28.5 gm of glycine and 6 gm of Tris in 100 ml of double distilled water and make it up to 1000 ml with double distilled water.
5. *Working reservoir buffer (pH 8.3)* : Dilute 60 ml of stock reservoir buffer to 600 ml with double distilled water.

6. *Running gel (7%)*: Mix small pore buffer, monomer, water and catalyst in the ratio of 1 : 2 : 1 : 4 (in volume). Fix vertical tubes of 70 × 5 mm size into the polymerising stand. Pour the running gel mixture into these tubes without introducing any air bubble. Place a drop of water over the gel without disturbing the gel layer. Polymerization is purely chemical and after ensuring the polymerization, remove the water layer at the end of 30 minutes.
7. *1% Amido black*: Dissolve 1 gm of amido black in 100 ml of 7% acetic acid.
8. *0.25% Coomassie brilliant blue*: Dissolve 250 mg of Coomassie brilliant blue in a solution containing methanol, water and acetic acid in the ratio of 5 : 5 : 1 (Smith, 1968).
9. *Saturated solution of oil red 'O'*: Saturated solution of oil red 'O' is prepared in 50% methanol containing 10% TCA (W/V) (Smith, 1968 ; Kannupandi and Paulpandian, 1975).
10. *Destaining solution*: 7% acetic acid (Dilute 7 ml of acetic acid to 100 ml with double distilled water).

3.4. PREPARATION OF SAMPLES

Directly collect a drop of haemolymph (0.034 ml) into a pre-chilled test tube containing 2 ml of 40% sucrose, after cutting the propodus or dactylus of one appendage so as to allow free bleeding. Collect fresh samples of *S. serrata* and *Emerita* ovary in different stages of its maturation and wash it with double distilled water to remove the adhering haemolymph. Wipe off the excess water by a filter paper.

Homogenize 10 to 20 mg of ovary in 40% sucrose, centrifuge and use the clear supernatant after removing the lipid cap.

3.5. PROCEDURE

1. Introduce the sample (haemolymph or ovary homogenate) in all maturation stages of ovary of *S. serrata* and *Emerita* in 40% sucrose over the gel layer. To prepare 40% sucrose dissolve 40 gm of sucrose in 100 ml of double distilled water.

2. Remove the gel tubes by giving slight lateral shakes without damaging the polymerized bottom of the gel.
3. Insert the gel tubes with the sample into the rubber grommets in the upper buffer chamber by screwing in after moistening the rubber grommets.
4. Fill the lower buffer chamber with 250 ml of working reservoir buffer and the upper buffer chamber with 250 ml of working reservoir buffer, with 2 drops of bromophenol blue.
5. Supply a constant current of 3 mA/tube until the bromophenol blue reached the gel edge. Electrophoresis was done inside the refrigerator.

3.6. STAINING

3.6.1. Simple proteins

Stain the gels in 1% amido black or 0.25% Coomassie brilliant blue. To stain in Coomassie brilliant blue prefix the gels in 10% TCA (Dissolve 10 gm of TCA in 100 ml of distilled water) for an hour.

Destaining : Repeatedly wash in 7% acetic acid and store in the same solution.

3.6.2. Complex proteins

Lipoproteins

Stain the gels in oil red 'O' for about 4 hours and store the gels in the same staining solution.

Glycoproteins

Leach the gels in 7% acetic acid for an hour. Wash in double distilled water for an hour. Fix the gels in 1% periodic acid for an hour. (Dissolve 1 gm of periodic acid in 100 ml of 3% acetic acid). Wash in double distilled water for an hour. Stain in Schiff's reagent for 30 minutes (For preparation of Schiff's reagent refer Expt. No. 2).

Destaining : Repeatedly wash in 1% sodium metabisulphite and store in the same. (Destaining reagent is prepared by dissolving 1 gm of sodium metabisulphite in 100 ml of double distilled water).

All the staining procedures should be made inside the refrigerator.

Haemocyanin

Prepare a saturated solution of dithio-oxamide (rubeanic acid) in 5:2:5 ratio of methanol, glacial acetic acid and water (Horn and Kerr, 1969).

Destaining : Destain and store the gels in 7% acetic acid.

3.7. CALCULATION OF R_m VALUES

After considerable destaining make the line drawings and calculate the relative mobility (R_m) of each fraction.

$$R_m = \frac{\text{Distance travelled by the protein fraction}}{\text{Distance travelled by the bromophenol blue}}$$

Find out the histochemical nature of each protein and tabulate them.

3.8. DENSITOMETRIC ANALYSIS

Scan the gels in a densitometer so as to quantify each protein.

3.9. OBSERVATIONS

i) Compare the electrophoretic mobility as well as the staining properties of different protein fractions of the blood of male and female. Also compare the blood of mature female with the proteins of ovary in different stages of maturation in *Emerita* and *Scylla*.

ii) Observe the newly appearing proteins in the blood and their homologous bands in the ovary.

iii) Compare the electrophoretic mobility and histochemical characteristics of the sex limited protein of *S. serrata* with that of *E. asiatica* and other known decapod crustaceans.

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**SEROLOGICAL IDENTIFICATION OF VITELLOGENIN
AND LIPOVITELLIN IN *SCYLLA SERRATA* AND
EMERITA ASIATICA USING IMMUNO
ELECTROPHORESIS***

4.1. INTRODUCTION

Vitellogenin is the blood protein precursor of lipovitellin, the main yolk protein. Many crustacean workers have used electrophoresis to detect the vitellogenin and lipovitellin in the blood and ovary respectively. However, authenticity of identical relative mobilities in homologizing vitellogenin with lipovitellin has been sometimes questioned because fluctuating current, buffer strength and gel composition may lead to variations in the relative mobility of the same component. It is therefore necessary to confirm the results of electrophoresis by serological investigations. Immuno-electrophoresis has been tried for studying the relationship between lipovitellin and vitellogenin in a few crustacean forms (Kerr, 1969 ; Croisille *et al.*, 1970 ; Ezhilarsi, 1982). Many of the crustacean blood proteins are immunogenic and produce antibodies in the mammalian blood. Therefore, cross reaction of these antibodies with the suspected identical proteins will produce specific precipitation arcs thus enabling real comparison. In addition, this method is advantageous in tracing the origin of vitellogenin into the extraovarian sources, even if the concentration of such precursors is very low.

4.2. PRINCIPLE

Antibodies to vitellogenin and lipovitellin are raised by active immunization by injecting the antigen directly to albino rabbits. Antigen-antibody reaction is carried out in the gelified medium either in agarose/agarose or polyacrylamide/agarose and bands of precipitation form wherever an antibody and its corresponding

* Prepared and verified by S. Ezhilarsi and T. Subramoniam, unit of Invertebrate Reproduction, Department of Zoology, University of Madras-600 005.

antigen meet at the optimal proportion. Both vitellogenin and lipovitellin of *S. serrata* are immunogenic in that, they give rise to antibodies that will specifically react with them. When these soluble antigen and antibody diffuse towards each other in a gel, a precipitin line is formed at their place of meeting and form an impermeable barrier to the antigen and antibody that has formed it. This is permeable to all other substances that are not concerned with that precipitate in question. However, this barrier persists only as long as some of the forming ingredients are present in the gel on either side of it.

4.3. PROCEDURE

4.3.1. Preparation of antiserum

4.3.1.1. Rabbit anti-lipovitellin antiserum

1. Homogenize 500 mg of freshly laid eggs of *S. serrata* and *E. asiatica* (0.0167 gm of protein) in *Carcinus maenas* Ringer solution (Smith and Ratcliffe, 1978). To prepare *C. maenas* Ringer solution dissolve 33.7 gm sodium chloride, 0.94 gm potassium chloride, 2.83 gm calcium chloride, 5.38 gm magnesium chloride, 0.193 gm disodium hydrogen phosphate and 6.060 gm Tris (hydroxymethyl) methylamine in 42.5 ml of 1 M hydrochloric acid and make it upto 1 litre. The pH is adjusted to 7.4.

2. Centrifuge at 5,000 g and decant the supernatant without the lipid cap. This clear supernatant will serve as the source of antigen.

3. Mix 1 ml of antigen and an equal volume of Freund's complete adjuvant (Difco).

4. Inject this mixture subcutaneously on 1st, 14th, and 21st days.

5. Third injection is a booster injection having double the quantity of antigen.

4.3.1.2. Rabbit anti-haemolymph containing female specific protein (FSP) antiserum

1. Collect 2 ml of haemolymph containing FSP (0.9074 gm protein) from *S. serrata* in late IVth stage or early Vth stage of ovarian maturation. Add this to a mixture containing 1 ml.

of 12.5% sodium citrate and 1ml of *C. maenas* Ringer solution (To prepare sodium citrate solution dissolve 12.5 gms of it in 100 ml of double distilled water).

2. Mix 2 ml of haemolymph with 2 ml of Freund's complete adjuvant and inject into the rabbits subcutaneously as described above.

4.3.1.3. Rabbit anti-control antiserum

Prepare the carrier medium by mixing 0.67 ml of *C. maenas* Ringer with 0.33 ml of 12.5% sodium citrate. Mix 1 ml of this with 1 ml of Freund's complete adjuvant and inject subcutaneously.

4.3.2. Collection of antiserum

1. Collect rabbit blood by puncturing ear vein on 27th day. Collect blood in the same way on 34th day also, into a sterilized boiling test tube.

2. Plug the tubes with cotton and leave it overnight in the refrigerator slantingly.

3. Collect the separated serum into sterilized screw cap vials and store in refrigerator.

4.3.3. Purification of antiserum

1. Mix 1 ml of antiserum with 1 ml of freshly collected male haemolymph in *C. maenas* Ringer.

2. Centrifuge at 6,780 g for 10 minutes to precipitate the reaction products formed by the interaction between the common antigens of male *S. serrata* and *E. asiatica* haemolymph and antibodies of female haemolymph containing FSP antiserum.

3. Mix the supernatant with 1 ml of male haemolymph repeatedly after each centrifugation, till no visible precipitate was formed (Fyffe and O'Connor, 1974).

4.3.4. Preparation of agarose gels

1. Prepare 1 litre of 0.015 M Tris-barbital buffer at pH 8.8 using a glass electrode pH meter.

2. Boil 1 gm of agarose in 100 ml of 0.015 M Tris-barbital buffer at pH 8.8 with 0.1 mg of methiolate. Methiolate avoids bacterial growth.

3. Evenly spread 2 ml of this mixture on a clean microscopic slide and allow to solidify.

4. Make a central trough and two side wells prior to the application of antigen.

4.3.5. Preparation of Polyacrylamide gels (*vide* Expt. No. 3.)

4.3.6. Separation of antigen proteins by agarose gel electrophoresis (Fig. 1)

1. Remove the slice of agarose on the well and deposit haemolymph collected in 40% sucrose or ovary homogenized in 40% sucrose (haemolymph and the ovary of *S. serrata* and *E. asiatica* are collected from different stages of ovarian maturation).
2. Before application mix the samples with the marking dye, bromophenol blue.
3. Fill the electrophoretic tank with 0.06 M Tris barbital buffer at pH 8.8. Place agarose smeared glass slides in the tank.

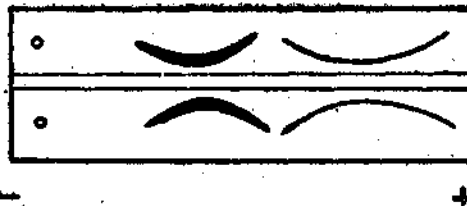


Fig. 1.

Immunoelectrophoresis: Both fractionation and diffusion of antigen is carried out in agarose: *Antigen*: Female matured *S. Serrata* haemolymph containing FSP. *Antibody*: Rabbit anti FSP antiserum.

Note the thick precipitate arc formed against FSP. Thin precipitin arc corresponds to haemocyanin fractions. (Slow moving fractions are not represented).

4. Prepare two paper wicks with Whatman No. 1 filter paper and place it on the two edges of the glass plate dipping into the buffer solution, so that the gel is connected to the buffer by the paper wicks.
5. Supply 1.6 mA/slide till the marker dye reaches the anodic end of the slide.
6. After the completion of electrophoresis switch off the current, remove the paper wick and slide.
7. Remove the agarose gel in the centre to have a longitudinal groove and procure appropriate immuno serum into it.
8. Incubate the slides in a humid chamber to allow immuno-diffusion at 34°C for 24 hours.

4.3.7. Separation of antigen proteins by polyacrylamide gel electrophoresis

(vide Expt. No. 3 ; Fig. 2).

4.3.8. Transfer method (Fyffe and O'Connor, 1974)

1. Transfer the polyacrylamide gels containing the resolved antigenic proteins onto a slide.
2. Mix 0.5 ml of antiserum with two ml of agarose-buffer mixture and pour it around polyacrylamide gel.
3. Incubate the slides in a humid chamber to allow immuno-diffusion at 34°C for 24 hours.
4. Note the precipitin arcs developed as white curved lines after the incubation period (Fig. 3).
5. Wash the gels with 1% saline for about 16 to 20 hours with frequent changes of saline and wash finally in double distilled water for an hour to remove saline.
6. Place the gel slides horizontally and filter paper strips over the gel to absorb excess water in the gel. After drying, filter paper comes out of the gel of its own.
7. Stain the gels with 0.1% amido black (W/V) for 10 minutes and destain them in 2% acetic acid till the gel becomes transparent and the arcs are clearly visible.



Fig. 3.

Polyacrylamide disc fractionation of *S. serrata* haemolymph and ovarian proteins.

1. Male *S. serrata* haemolymph. 2. Female immature *S. serrata* haemolymph. 3. Female maturing *S. serrata* haemolymph. 4. Female mature *S. serrata* haemolymph. Arrow indicates the appearance of female-specific protein (FSP) in late stage IV and early stage V. 5. Mature ovarian proteins of *S. serrata*. Note the homologous proteins in the ovary and haemolymph.



Fig. 3.

Immunoelectrophoresis: Transfer method. Antigens separated on polyacrylamide is diffused through agarose containing antiserum.

1. Female *S. serrata* containing FSP \times Rabbit anti haemolymph containing FSP antiserum. 2. Female *S. serrata* containing FSP \times Rabbit antilipovitellin antiserum. 3. Freshly laid egg proteins of *S. serrata* \times Rabbit anti haemolymph containing FSP antiserum. 4. Freshly laid egg proteins of *S. serrata* \times Rabbit anti-lipovitellin antiserum. (Slow moving fractions are not represented as they are not immunogenic).

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EXPERIMENTS ON YOLK PROTEIN UPTAKE IN CRUSTACEAN OVARY*

5.1. INTRODUCTION

Recent electron microscopic and biochemical investigations have revealed the pinocytotic uptake of extra ovarian proteins into the vitellogenic oocytes (Hinsch and Cone, 1969 ; Wolin *et al.*, 1973). The uptake of these macromolecular yolk precursor substances could be demonstrated using trypan blue as well as horse radish peroxidase.

5.2. MATERIALS

Ovaries of *Scylla serrata* and *Emerita asiatica* in different stages of maturation.

5.3. TRYPAN BLUE METHOD

5.3.1. Principle

Trypan blue dye used as the indicator in the present experiment mimics the physical properties of proteins and the dye molecules are too big to enter living cells except by pinocytosis. Hence, incorporation of trypan blue into the oocyte indicates its ability to sequester extra ovarian macromolecular proteins.

5.3.2. Procedure

1. Dialyse trypan blue against water.
2. Prepare 1% trypan blue solution in saline.
3. Dissect out ovaries of *S. serrata* and *E. asiatica* in different stages of ovarian maturation under sterile conditions.

* Prepared and verified by S. Ezhilarasi, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

4. Wash in saline to remove the adhering haemolymph.
5. Incubate the ovaries in 1% trypan blue solution for 1 hour.
6. Wash the ovaries in four changes of saline for 1 hour.
7. Prepare the ovaries for cryocut sectioning.
8. Examine the sections of the ovaries under microscope.
9. Observe and differentiate the rate of micropinocytosis of trypan blue in different stages of ovarian maturation.

5.4. HORSE RADISH PEROXIDASE METHOD

5.4.1. Principle

Horse radish peroxidase is an enzyme whose presence can be detected histochemically using hydrogen peroxide and benzidine. When a solution of peroxidase is injected into the haemolymph of crustaceans these macromolecules would be sequestered by a tissue or cell involved in pinocytosis. The tissue, after appropriate incubation, can be processed for benzidine reaction. Since the vitellogenic oocytes are known to sequester the yolk precursors during vitellogenesis the peroxidase method could be used to demonstrate the uptake of protein molecules into the oocytes. This method has been previously used to demonstrate the protein sequestration into the fat body of insects and millipedes (Lock and Collins, 1968 ; Subramoniam, 1971).

5.4.2. Procedure

1. Prepare 1% solution of horse radish peroxidase in normal saline.
2. Inject 1 ml of horse radish peroxidase solution into the crab.
3. After 4 hours dissect out the ovaries and fix in cold 4% neutral buffered formalin for 4 hours.
4. Wash in 10% sucrose solution 3 times at an interval of 30 minutes.
5. Wash in phosphate buffer (pH 7).

6. With gentle shaking, add 9 ml of benzidine reagent (dissolve 300 mg of benzidine in phosphate buffer at pH 7).
7. After 2 minutes add 1 ml of hydrogen peroxide and shake vigorously at room temperature for 10-30 minutes.
8. Prepare the tissues for cryocut sections.
9. Observe the dark brown granules in the ooplasm for the presence of peroxidase.

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ESTIMATION OF CAROTENOIDS IN THE OVARY OF THE EDIBLE CRAB *SCYLLA SERRATA**

6.1. INTRODUCTION

Carotenoids, the yellow-red pigments found in plants and animals are $C_{40}H_{56}$ compounds with 8 polyene isoprenoid residues, aliphatic or alicyclic in structure and with many conjugated double bonds. β -carotene is the starting point for the range of carotenoids in the animals. These are primordial plant pigments evolved along with the chlorophyll system acting as an accessory light-harvesting system not known to be synthesised by animal tissues. Entering animals exclusively through food, β -carotenes may be oxidized in the tissues into keto-carotenoids (Cheeseman *et al.*, 1967) best exemplified in the ovarian tissue of decapods. The normal pathway is β -carotene \longrightarrow isocryptoxanthin \longrightarrow echinenone \longrightarrow canthaxanthin \longrightarrow astaxanthin. In the Brachyura, the carotenoids may also be deposited in protein complexes in the integument or may be dissolved in lipids in the ovary and hepatopancreas or form carotenoglycolipoprotein complexes in the hemolymph (Zagalsky *et al.*, 1967). In *Eupagurus bernhardus* and *Clibanarius erythropus*, astaxanthin-protein complexes are found in the exoskeleton (Goodwin, 1952). In *Emerita analoga*, Gilchrist and Lee (1972) found a differential distribution of carotenoids in the different coloured eggs. Eg. Orange eggs (stage 1) have lipocarotenoprotein complexes while brown eggs (stage 2) have carotenoprotein complexes.

The function of carotenoids in decapod systems is as varied as the number of forms in which they are present. The unenolised keto groups of carotenoids are essential to complex formation. Experiments of Zagalsky *et al.* (1967) have shown carotenoids

* Prepared and verified by Sudha Varadarajan, Zoological Survey of India, Madras-600 004.

to act as a powerful lock to the tertiary and quaternary structure. Carotenoprotein complexes protect tissues against photodamage as well as lipid peroxidation (Mathews-Roth *et al.*, 1974). Carotenes can act as substitutes to vitamin A in invertebrate tissues. Ceccaldi and Martin (1969) suggested that carotenoids can act as carriers for other macromolecules from hepatopancreas to ovary. The tail fin-pattern in the penaeid post-larvae of the Madras brackish waters, provides a reliable criterion for identification of the post-larval stages (Muthu, 1978).

6.2. PRINCIPLE

Carotenoids, being lipids containing in solution coloured hydrocarbons, are soluble in light petroleum, ether, benzene, chloroform, carbon-tetrachloride, alcohol and acetone. Suspension of carotenoid-containing tissue in any of these solvents extracts carotenoids. Bound carotenoids are released from complexes by addition of protein denaturing agents followed by a chosen lipid solvent. The extract is diluted to a known volume before measuring the maximum absorption in that solvent for β -carotene (*Eg.* absorption at 450 nm in chloroform).

6.3. MATERIAL

1 gm of tissue in every developmental stage of ovary, egg and corresponding hepatopancreatic tissue.

6.4. REAGENTS (Olson, 1979)

1. *Anhydrous sodium sulphate*, reagent grade.
2. *Chloroform* stabilized with 0.75% absolute ethanol,
3. *Crystalline β -carotene* (E-Merck).

6.5. PROCEDURE

6.5.1. Sample Storage

Samples should be quickly removed from the animals, placed in small glass vials closed with teflon stoppers or with screw-caps lined with aluminium foil, frozen at -20°C and stored until analysis. Samples should be analysed within a week after storage.

6.5.2. Sample Extraction (Olson, 1979)

Quickly weigh 1 gm of tissue on a tared piece of aluminium foil to the nearest 10 mg, place in a 10 ml screw cap clean glass vial. Add 2.5 gm of anhydrous sodium sulphate. Gently mash the sample with a glass rod against the side of the vial until it is reasonably well-mixed with the sodium sulphate. Cover with 5 ml of chloroform. Seal the glass vial and place at 0°C overnight (8-24 hours). The chloroform should form a clear 1-2 cm layer above the caked residue. Prepare a blank in a similar manner.

6.5.3. Spectrophotometric Analysis

Take an aliquot of 0.3 ml of the chloroform extract and dilute to a volume of 3 ml with ethanol. Treat the blank in a similar manner. Transfer to 1 cm cuvette (4 ml capacity) and read absorption in a spectrophotometer at 290, 350, 380, 450, 475 and 500 nm. Plot the readings on a graph.

6.5.4. Calculation

The total carotenoid content is calculated as μg carotenoids gm tissue :

$$\frac{\text{Absorption at 450 nm} \times \text{dilution factor}}{0.25 \times \text{sample wt. (gm)}}$$

Dilution factor in this experiment : 50

0.25 = Extinction coefficient.

6.5.5. Comments on Procedure (Olson, 1979)

Mashing should not be done too well, the mixture of sample and sodium sulphate should never be ground to a fine powder because

- a) aerosol from the powder poses a health problem, and
- b) the chloroform layer may become cloudy with fine powdering interfering in assaying.

Use manual pipettes for all transfers. Do not use ground glass stoppered vials as they leak even if taped. Use only screw-cap vials and seal tubes hermetically to avoid loss of solvent during the 8-24 hr. extraction period.

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THIN LAYER CHROMATOGRAPHIC SEPARATION OF LIPIDS IN OVARY, TESTIS AND GUT OF THE SEA URCHIN *SALMACIS VIRGULATA**

7.1. INTRODUCTION

Sea urchins accumulate large amounts of lipid in ovary during its reproductive cycle (Giese, 1966 ; Vivek Raja, 1980). Lipids deposited in the developing gonads may be synthesized within the oocytes or transported from the gut. A variety of lipid classes are also found to occur in the ovary, testis, gut, body wall and coelomocytes of the sea urchins (Allen, 1974 ; Vivek Raja, 1980). The present experiment is designed to separate and identify the different lipid classes present in gut, testis and ovary of the sea urchin *Salmacis virgulata* employing thin layer chromatographic method.

7.2. PRINCIPLE

Chromatography is a separation process based on differential distribution between two immiscible phases one of which moves relative to the other. The element of motion may be provided not by movement of phase but by movement within a phase, as in electromigration. In thin layer chromatography (TLC) silica gel placed on the glass plate acts as stationary phase. The mixture of compounds to be separated is placed near one end of the TLC plate and allowed to dry. Then the TLC plate is placed into a chromatographic chamber containing a relevant solvent system (mobile phase). As the solvent travels towards the far end of the plate, by adsorption and capillary action, the test mixture separates into various components. When the solvent reaches the far end of the plate, the plate is removed, rapidly dried and the spots are detected using a suitable location reagent (Stahl, 1958 ; Smith and Ersser, 1976).

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt. Arts College, Nandanam, Madras-600 035.

7.3. MATERIALS

Immature and ripe ovary, testis and gut of sea urchin *Salmacis virgulata*.

7.4. REAGENTS

1. 0.9% Sodium chloride: Dissolve 900 mg of sodium chloride in 100 ml of distilled water
2. Silica gel-G
3. Solvent—I for neutral lipid separation: Diethyl ether—benzene—ethanol—acetic acid. Mix in the proportion of 40 : 50 : 2 : 0.2 (V/V)
4. Solvent II for neutral lipid separation: Diethyl ether—hexane (6 : 94; V/V)
5. Solvent for phospholipids separation: Chloroform—methanol—water (65 : 25 : 4; V/V)
6. Iodine crystals.
7. Standard neutral lipids.
8. Standard phospholipids.

7.5. PROCEDURE

7.5.1. Extraction of lipid (Folch *et al.*, 1957)

1. Take 500 mg of wet tissue in a homogenizer.
2. Add 10 ml of chloroform/methanol (2 : 1 V/V) solution and homogenize it well with teflon or glass homogenizer.
3. Filter the homogenate through a Whatmann No. 1 filter paper.
4. Add 2 ml of salt solution (0.9% aqueous sodium chloride) and shake well.
5. Transfer the mixture to a small separating funnel and allow it to stand overnight at 4°C.
6. A clear biphasic layer will form. The lower phase contains all the lipids. Remove the lower phase and adjust the volume to 10 ml by the addition of chloroform.

7. Take 5 ml in a watch glass and allow the solvent (chloroform) to evaporate at room temperature (preferably 50-60°C) for five hours. Then dry the same *in vacuo* over silica gel for 7 days. Weigh the lipid and calculate the percentage of lipid present in the tissue.
8. From the remaining 5 ml a known quantity (50-100 μ l) may be used for spotting on TLC plate using micro-pipette.

7.5.2. Preparation of plate

1. Mix 20 gm of silica gel-G with 40 ml of water and stir well until a fine slurry without lumps is obtained.
2. Adjust the TLC spreader to 0.3 mm thickness and pour the aqueous slurry into the spreader.
3. Draw the spreader from one end of the unit to the other end at an even rate over the glass plates (20 \times 20 cm).
4. Leave the plates to dry in air for 30 minutes.
5. Keep the plates horizontally in the oven at 105°C for 1 hour.
6. Remove the plates from the oven and keep them in room temperature for 15 minutes.
7. Once the temperature of the plates reduces to the room temperature it is ready for spotting.

7.5.3. Spotting (Application of sample on the TLC plate)

For the best separation and resolution the sample dissolved in chloroform-methanol is best applied in the form of a band rather than a spot. Single spots tend to 'tail'. The band, usually spread over 2-2.5 cm can be applied dropwise with a micropipette. The solvent may need evaporating from time to time to allow more sample to be added over a small area. A stream of nitrogen gas or hair drier can be used for this purpose. A sample applicator is more convenient and has the advantage of even loading (Lake and Goodwin, 1976).

7.5.4. Neutral lipids (Freeman and West, 1966)

1. Pour 100 ml of solvent—I (Diethyl ether—benzene—ethanol—acetic acid 40 : 50 : 2 : 0.2; V/V) in the tank and close it with the lid. Leave the tank without disturbance for 30 minutes.
2. Keep the spotted plate, vertically in the tank. Care should be taken that there is about 1 cm distance between the level of irrigating solvent and spots (*i.e.* point of application of sample) on the plate.
3. Allow the irrigation solvent to run a distance of 12 cm from the spot (origin).
4. Remove the plate from the tank, allow it to dry in room temperature for 15 minutes and keep it in oven at 40°C for 5 minutes to remove the traces of acetic acid.
5. Then keep the plate in another tank containing 100 ml of solvent II (Diethyl ether—Hexane, 6 : 94 V/V).
6. Allow the solvent to run up to 15 cm from the spot.
7. Remove the plate from the tank and dry it at 60°C for 30 minutes.
8. Keep the plates in a glass chamber with iodine vapour. Clear spots will appear on the plate. Take photograph and calculate the R_f values :

$$R_f = \frac{\text{distance substance travels from the origin}}{\text{distance solvent front travels from the origin}}$$

Compare the R_f values of sample with the standard neutral lipids run in the same plate or in different plates in the identical condition.

7.5.5. Phospholipids (Allen, 1974)

1. Pour 100 ml of chloroform—methanol—water (65 : 25 : 4; V/V) in the tank and close it with the lid. Leave the tank undisturbed for 30 minutes.
2. Keep the plate in the tank and allow the irrigation solvent to run a distance of 16 cm from the spot.

3. Remove the plate from the tank and dry at 60°C for 30 minutes.
4. Keep the plate in a glass chamber with iodine vapour. Clear spots appear on the plate. Take photograph and calculate the R_f values.

7.6. OBSERVATION

Identify the neutral and phospholipid fractions by comparing the R_f values of the samples with the standard lipids, developed under the identical conditions. The carotenoid pigments exhibit yellow colour before the treatment with the iodine vapour. Hence they should be marked and identified before keeping the TLC plate in the iodine vapour. Draw the line diagram of the TLC plate showing the different lipid fractions.

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III. SPERM MORPHOLOGY AND SPERMATOPHORES OF CRUSTACEA

IN VITRO OBSERVATION ON SPERM MORPHOLOGY IN A FEW DECAPOD CRUSTACEANS*

8.1. INTRODUCTION

Based on the classification of Afzelius (1971), the spermatozoa of marine invertebrates can be categorized into 1) primitive 2) simplified 3) modified and 4) atypical type. He has also correlated the morphological features of the spermatozoa with the type of fertilization. Decapod crustaceans are peculiar in possessing non-flagellate, vesiculiform spermatozoa (Nath, 1956). Among the different groups of decapods, the morphology of the spermatozoa is highly variable and is species-specific. The organisation of these vesiculiform spermatozoa also differs from that of the typical spermatozoa in that it is not possible to discern the typical structures such as head, mid piece and tail. In general, the acrosomal vesicle, under light microscopic observation, is found to be present in the centre of the sperm cells, when viewed from above. The mitochondria are completely fragmented and are found mixed with chromatin granules, forming the outermost layer, namely nuclear-mitochondrial nebenkern. In between the central acrosomal vesicle and the nuclear mitochondrial nebenkern are found two vesicles (primary and secondary). Though immotile, decapod spermatozoa possess variable numbers of rays or spikes. These structures are not to be confused with the flagellum of the typical spermatozoan, as they lack the 9 + 2 filamental pattern.

8.2. MATERIALS

Live specimens of *Emerita asiatica*, *Albunea symnista*, *Clibanarius longitarsus*, *Penaeus indicus* and *Scylla serrata*.

* Prepared and verified by K. Uma, Unit of Invertebrate Reproduction Department of Zoology, University of Madras, Madras-600 005.

8.2.1. Reagents

1. 1% *Neutral red* : Dissolve 1 gm of neutral red in 100 ml of distilled water.
2. 1% *Congo red* : Dissolve 1 gm of Congo red in 100 ml of distilled water.
3. 0.1% *Acetocarmine* : Dissolve 100 mg of acetocarmine in 100 ml of distilled water.
4. 0.1% *Methylene blue* : Dissolve 100 mg of methylene blue in 100 ml of distilled water.
5. 0.1% *Fast green* : Dissolve 100 mg fast green in 100 ml of distilled water.
6. 0.1% *Basic fuchsin* : Dissolve 100 mg of basic fuchsin in 100 ml of distilled water.
7. 0.1% *Janus green* : Dissolve 100 mg of Janus green in 100 ml of distilled water.
8. 0.1% *Bromophenol blue* : Dissolve 100 mg of bromophenol blue in 100 ml of distilled water.
9. 1% *Periodic acid* : *Vide* section 2.5.1.
10. *Schiff's reagent* : *Vide* section 2.4.3.

8.3. METHOD

8.3.1. Staining with vital dyes

1. Dissect the specimen and separate the male reproductive system.
2. Cut a bit of the distal portion of the vas deferens to separate the spermatophoric components.
3. Transfer to a clean slide and apply a coverslip on it and press gently.
4. Add a few drops of prepared vital stain (*e.g.* 0.1% methylene blue) at the edges of coverslip and allow them to stain the material for a few minutes.
5. Wipe off the excess stain before observation.

The same procedure is used for different materials with different stains.

8.3.2. Periodic acid-Schiff method (Pearse, 1968)

1. Treat the prepared fresh smear in 1% periodic acid for 10 minutes.
2. Wash in running water for 5 minutes.
3. Immerse in Schiff's reagent for 10 minutes.
4. Wash in running water for 5 minutes.
5. Observe the intensity of magenta colour.

8.4. OBSERVATION

After staining with the vital dyes, describe the morphology of the spermatozoa of the crustaceans given above. Find out the differences in the number as well as the shape of the spikes/rays. Also, distinguish and characterize the various organelles from specific staining reaction. PAS positivity is obtained in the acrosomal region. Nuclear mitochondrial nebenkern will specifically stain with Janus green and the nuclear stains. The vesicles will remain unstained.

8.5. REFERENCES

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SPERM EXPLOSION (DEVAGINATION) PROCESS IN DECAPOD SPERMATOOA*

9.1. INTRODUCTION

The mature, vesiculiform spermatozoa of decapods are known to undergo certain structural changes due to the lowering of osmotic pressure in the medium which surrounds the spermatozoa (Fasten, 1921). Differences in the electrolytes of the medium would also lead to sperm explosion (Brown, 1966). This process is otherwise known as evagination or eversion (Rathnavathy, 1947) and devagination (Pochon-Masson, 1968). During this process, the structural changes vary in different types of spermatozoa. The evagination process observed in the decapod spermatozoa is shown to be a prerequisite for sperm-egg interaction during fertilization (Binford, 1913 ; Brown, 1966 ; Pochon-Masson, 1968).

9.1. MATERIALS

Scylla serrata, *Albunea symnista* and *Clibanarius longitarsus*.

9.3. REAGENTS

1. 2% *Sodium chloride* : Sodium Chloride 2 gm in 100 ml of distilled water.
2. 2% *Potassium chloride* : Potassium Chloride 2 gm in 100 ml of distilled water.
3. 0.1% *Potassium hydroxide* : Potassium hydroxide 100 mg in 100 ml of distilled water.

9.4. PROCEDURE

1. Prepare fresh smears of seminal substances to observe the structure of spermatozoa.

* Prepared and verified by K. Uma, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

2. Treat them with distilled water for 5 minutes and observe the structural changes of spermatozoa under higher magnification.

9.5. OBSERVATION

Find out the structural alterations of organelles such as acrosome vesicles, nuclear mitochondrial nebenkern with the various solutions. Treat the spermatozoa with sea water and the reagents listed above. Record the sequential changes occurring during the process of evagination with the aid of camera lucida. Devagination process in the sperm of *S. serrata* is given in Fig. 1.

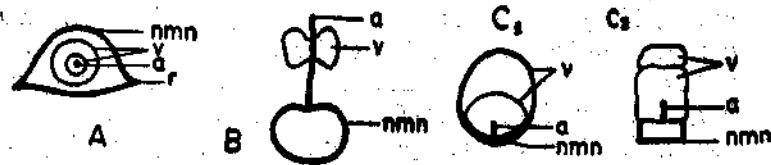


Fig. 1.

Process of devagination in the sperm of *Scylla serrata*. A—normal structure; B—devaginated—after treatment with water; C1 and C2—devaginated—after treatment with 2% salt water. A—acrosome; nmh—nuclear-mitochondrial nebenkern (nuclear cup) (Fasten, 1921).

9.6. REFERENCES

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**A MORPHOLOGICAL INVESTIGATION ON
THE SPERMATOPHORES OF SELECTED
CRUSTACEANS***

10.1. INTRODUCTION

In many decapod crustaceans, the male produces discrete aggregations of spermatozoa embedded in some form of protective covering, termed the spermatophores. They are transferred during mating to the oviduct or merely deposited on the sternum of the females. In this way, the spermatozoa can be retained in a viable state by the female until such time the ova are ready for fertilization. In Malacostraca, spermatophores assume a variety of shapes, and show variation in their chemical composition (Malek and Bawab, 1971 ; Uma and Subramoniam, 1979 ; Subramoniam, 1982). A correlation between the spermatophore morphology and the type of fertilization has been suggested by Spalding (1942), Uma and Subramoniam (1979) and Subramoniam (1982) for Crustacea. The present experiment aims at studying the variations occurring in the spermatophore morphology of representative crustaceans with the idea that a classification for crustacean spermatophores may be arrived at.

10.2. MATERIALS

Scylla serrata, *Clibanarius longitarsus*, *Emerita astatica*, *Albunea symmista*, *Penaeus indicus* and *Ligia exotica*.

10.3. REAGENTS

0.1 % Methylene blue : *Vide* expt No. 8.

0.1 % Fast green : *Vide* expt. No. 8.

* Prepared and verified by K. Uma and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

10.4. PROCEDURE

10.4.1. *Scylla serrata*

1. Dissect the crab and remove the mid vas deferens (MVD).
2. Collect the contents from the MVD in a watch glass.
3. Pipette out the spermatophores, settled at the bottom, and then transfer to a slide.
4. Apply a coverslip on the material, add a few drops of stain at the edges of the coverslip. Observe under microscope after 5-10 minutes.

10.4.2. *Clibanarius longitarsus*

1. Open the shell, dissect the crab and remove the reproductive system.
2. Cut a small portion of distal vas deferens. Press one end of the vas deferens using a needle and remove the spermatophoric ribbon. Keep it on a slide.
3. Add a few drops of 0.1% methylene blue. Allow it to stain for 10 minutes and apply a coverslip. Observe under microscope.

10.4.3. *Emerita asiatica*

1. Pull the fifth leg at its base with a fine forceps. This results in the removal of the entire male reproductive organs without damage (Subramoniam, 1977). Transfer it to a slide and add a drop of water. Cut the hind portion of distal vas deferens. Press one end of the cut portion using a forceps to separate the spermatophoric ribbon.
2. Stain the spermatophoric ribbon for 5 minutes. Apply a coverslip and observe under microscope.

10.4.4. *Albunea symnista*

1. Remove the carapace carefully.
2. Trace the reproductive system and separate the bulged portion of distal vas deferens. Place it in distilled water and the spermatophoric mass comes out.

3. Transfer the spermatophoric mass to a glass slide, and stain for five minutes.
4. Likewise, remove the spermatophoric mass from the distal end of the vas deferens of *Penaeus indicus* and stain.

10.4.5. *Ligia exotica*

1. Pin the animal on the board and dissect to remove the reproductive system.
2. Cut the hind part of the vas deferens and transfer it to a slide. Shake the cut end in a slide to collect the contents alone. Add a few drops of stain immediately. Apply a coverslip and observe under microscope.

10.5. OBSERVATION

Compare the morphology of spermatophores of the crustaceans you have examined. Make a classification of spermatophores based on features such as peduncle and accessory mucoid secretions. Discuss the morphological features in relation to mode of fertilization and sperm transfer.

10.6. REFERENCES

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PERMEABILITY STUDIES AND DEHISCENCE OF SPERMATOPHORES*

11.1. INTRODUCTION

The mechanism of release of sperm from crustacean spermatophores has long been debated. Many factors such as external physical pressure, imbibition of water by substances within and an oviducal secretion have been suggested to be responsible for the opening up of spermatophores (Mouchet, 1931; Bloch, 1935; Subramoniam, 1977). In other forms, such as lobsters where the spermatophore is in the form of a complex gelatinous ribbon, the powerful chelae of the fifth leg is used for breaking the spermatophore and then gouges it open, thus releasing the spermatozoa (Fielder, 1964). In *Scylla serrata*, the free spermatozoa are seen in the spermathecal smear only at the spent stage of female (Ezhilarasi & Subramoniam, 1982). This suggests that the entry of spermathecal or ovarian fluid into the spermatophores may bring about the dissolution of spermatophore layers and spermatophoric substances. In the light of the above observations, permeability experiments using acids, alkalies and vital dyes have been made on the spermatophores of *Scylla serrata*.

11.2. PERMEABILITY STUDIES ON SPERMATOPHORES

11.2.1. Reagents

1. Glacial acetic acid
2. Concentrated hydrochloric acid
3. Concentrated sulphuric acid
4. 4% Sodium chloride: 4 gm of sodium chloride in 100 ml of distilled water.

* Prepared and verified by K. Uma and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

5. 2% *Potassium hydroxide* : 2 gm of potassium hydroxide in 100 ml of distilled water.

6. 4% *Sodium hydroxide* : 4 gms of sodium hydroxide in 100 ml of distilled water.

11.2.2. Procedure

1. Collect the freshly separated spermatophores in a cavity slide.
2. Treat the spermatophores with weak acids, strong acids, sodium chloride solution and alkaline solutions.
3. Tabulate the results

Chemicals	Changes occurring in the spermatophores
Acetic acid	
Conc. hydrochloric acid	
Conc. nitric acid	
4% Sodium chloride	
2% Potassium hydroxide	
4% Sodium hydroxide	

11.3. DEHISCENCE OF SPERMATOPHORES

11.3.1. Reagents

1. 0.1% Basic fuchsin
2. 0.1% Congo red
3. 0.1% Toluidine blue
4. 0.1% Methylene blue

(For preparation of the above solutions *vide* section 8.2.1)

11.3.2. Procedure

1. Add 0.1% solutions of basic fuchsin, toluidine blue, methylene blue and congo red to the spermatophores.
2. Observe the spermatophores using compound microscope after 5 minutes, 10 minutes and 15 minutes and note the colour intensity of layers and sperm mass.

3. Record the staining reactions of the spermatophore layers in the Table given below.

Dyes	5 minutes		10 minutes		15 minutes	
	SL	SM	SL	SM	SL	SM
0.1 % Basic fuchsin						
0.1 % Congo red						
0.1 % Toluidine blue						
0.1 % Methylene blue						

if there is no colour (—) ; if there is light colour (+) ; if the colour is intense (++) ; SL—Spermatophore layer ; SM—Sperm mass.

11.4. REFERENCES

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IV. BIOCHEMISTRY OF SEMINAL SECRETIONS

BIOCHEMICAL ANALYSES OF SEMINAL PLASMA AND SPERMATOPHORES OF *SCYLLA SERRATA**

12.1 INTRODUCTION

A volume of information is available on the nature, origin and role of seminal plasma in mammals (Mann, 1964 ; Hafez, 1976). Among invertebrates such information is limited to insects and echinoderms only. Cirripedes are the only crustacean group that has received sufficient attention on the biochemistry of seminal plasma (Barnes and Blackstock, 1974). Decapods, including many economically important crustacean species, have not received any attention with regard to seminal biochemistry. Hence, the commercially important brachyuran crab, *S. serrata* has been chosen for the analysis of important chemical components of seminal secretion. As the crabs of bigger size range are found to contain more quantity of seminal secretion in the mid vas deferens (Uma, 1982), only the crabs in the size range of 14-16.5 cm are used.

12.2 METHOD OF COLLECTION OF SEMINAL PLASMA AND SPERMATOPHORES

1. Separate the mid vas deferens from the reproductive system of crabs (14-16.5 cm) and keep them in a Petri-dish.
2. As the seminal contents coagulate immediately, pipette out the luminal contents using a micropipette into a centrifuge tube.
3. Centrifuge the contents for 5 minutes at 3000 rpm at 4°C to separate the seminal plasma and spermatophores. The

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spermatophores will sediment along with a small quantity of seminal fluid.

4. To separate and wash the spermatophores, mix the bottom sediment with distilled water and centrifuge for 3—5 minutes at 3000 rpm.

12.3. ESTIMATION OF PROTEIN (Lowry *et al.*, 1951)

12.3.1. Principle

The carbamyl groups of protein react with the copper ion of the alkali and when this complex reacts with phosphomolybdic acid of Folin reagent get reduced with tyrosine and tryptophan.

12.3.2. Reagents

1. 80% *Ethanol*: Dilute 80 ml ethanol with 20 ml of double distilled water.
2. 0.1 *N Sodium hydroxide*: 400 mg of sodium hydroxide in 100 ml distilled water.
3. 1 *N Sodium hydroxide*: 4 gms of sodium hydroxide in 100 ml of double distilled water.
4. *Solution A*: 2 gm of sodium carbonate in 100 ml of 0.1 *N* sodium hydroxide.
5. *Solution B*: 500 mg of copper sulphate in 1% sodium tartrate. (1 gm of sodium tartrate in 100 ml of double distilled water).
6. *Solution C*: Mix 50 ml of solution A with 1 ml of solution B and treat that as alkaline copper solution.
7. *Folin-ciocalteu phenol*: Mix 1 ml of Folin phenol with 1 ml of double distilled water.
8. *Standard*: Dissolve 1 mg of bovine serum albumin in 10 ml of 1 *N* sodium hydroxide and make up to 100 ml in a standard flask.

12.3.4. Procedure

1. Take 0.1 ml of seminal plasma and add 1 ml of 80% ethanol to precipitate the protein.

2. Centrifuge for 5 minutes at 4000 rpm to get a clear supernatant.
3. Dissolve the precipitate in 1*N* sodium hydroxide and make up to 10 ml with the same.
4. Take 1 ml from this and treat with 5 ml of solution C for 10 minutes.
5. Add 0.5 ml of Folin-ciocalteu reagent.
6. Read the colour intensity after 20 minutes at 700 nm.
7. Standard—From the standard take 1 ml.
8. Blank—Take 1 ml of 1*N* sodium hydroxide.

12.3.5. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu\text{g of standard} = \mu\text{g of protein in ml of seminal plasma}$$

12.4. ESTIMATION OF CARBOHYDRATE (Roe, 1955)

12.4.1. Principle

Sulphuric acid hydrolyses the di- and oligosaccharides into monosaccharides and converts the monosaccharides into furfural or furfural derivatives, which react with anthrone and produces a complex coloured product.

12.4.2. Reagents

1. 80% *ethanol*
2. *Anthrone reagent*: Dissolve 50 mg of anthrone powder in 100 ml of 66% sulphuric acid, to this add 1 gm of thiourea to stabilize the colour.
3. *Standard*: Dissolve 1 mg of glucose in 10 ml saturated benzoic acid.

12.4.3. Procedure

1. Take 1 ml of the sample, add 1 ml 80% ethanol; centrifuge it. Take 0.5 ml of clear supernatant and add 5 ml of

anthrone. Keep the tubes in the boiling water bath for 15 minutes.

2. Bring the tubes to the dark to protect from the light.
3. Record the absorbancies at 620 nm.
4. Standard—Take 0.5 ml of standard.
5. Blank—Take 0.5 ml of 80% ethanol.

12.4.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu\text{g of standard} = \mu\text{g of carbohydrate in ml seminal plasma}$$

12.5. ESTIMATION OF LIPID

12.5.1. Principle

The quantitative determination of lipid by sulphophosphovanillin method depends on the reaction of lipids extracted from the sample using chloroform - methanol, with sulphuric acid, phosphoric acid and vanillin to give a red complex.

12.5.2. Reagents

1. *Chloroform : methanol (2 : 1)* : Mix 20 ml of chloroform with 10 ml of methanol.
2. *0.9% sodium chloride* : Dissolve 900 mg of sodium chloride in 100 ml of distilled water.
3. *Phosphovanillin reagent* : Add 800 ml of orthophosphoric acid to 200 ml of distilled water. Dissolve 2 gm of vanillin in this solution.
4. *Standard* : Dissolve 8 mg of cholesterol in 4 ml of chloroform : methanol (2 : 1).

12.5.3. Procedure

1. Take 1 ml of seminal plasma and extract the lipid following the method of Folch *et al.* (1957).
2. To 1 ml of seminal plasma, add 1 ml of methanol and 2 ml of chloroform and to which add again 2 ml of chloroform methanol (2 : 1 V/V). Mix thoroughly.

3. To this add 0.2 volume of 0.9% sodium chloride solution. Pour this into a separating funnel and mix it. Allow it to stand for few hours.
4. Separate the lower phase into a clean tube. Make up the volume of the lower phase to the original quantity of chloroform added before.
5. To estimate the lipid quantity (Barnes and Blackstock, 1973) measure 0.5 ml of extract into a clean test tube. Allow it to dry in a vacuum desiccator over silica gel; dissolve in 0.5 ml of concentrated sulphuric acid. Mix well. Plug the tubes with non-absorbant cotton wool. Place in a boiling water bath for 10 minutes. Cool the tubes to room temperature.
6. Take 0.2 ml of this acid digest in a separate tube; add 5 ml of vanillin reagent. Mix well and allow to stand for half an hour. Measure the developed colour at 520 nm.
7. Standard—Take 0.2 ml of standard.
8. Blank—Take 0.2 ml of chloroform, allow it to evaporate.

12.5.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu\text{g of standard} = \mu\text{g of lipid in ml seminal plasma}$$

12.6. ESTIMATION OF ASCORBIC ACID (Roe, 1961)

12.6.1. Principle

The ascorbic acid is converted to dehydro-ascorbic acid by shaking with Norit and this is coupled with 2—4 dinitrophenyl hydrazine in the presence of thiourea as a mild reducing agent. Sulphuric acid then converts the dinitrophenyl hydrozone into a red compound which is analysed colorimetrically.

12.6.2. Reagents

1. 6% TCA : Dissolve 6 gm of TCA in 100 ml of distilled water.
2. 4% TCA : Dissolve 4 gm of TCA in 100 ml of distilled water.

3. *Acid washed Norit*: Place 200 gm of Norit in a large flask and add 1 litre of 10% HCl. Heat to boiling; then filter with suction. Remove the cake of Norit to a large litre of double distilled water, stir thoroughly, and filter. Repeat this procedure once. Dry the Norit overnight in an oven at 100–120°C.
4. *Dinitrophenyl hydrazine—Thiourea reagent*: Dissolve 2 gm of 2, 4—dinitrophenyl hydrazine in 100 ml 9N H_2SO_4 (3 parts of H_2O to 1 part of concentrated H_2SO_4). Add 4 gm of reagent grade thiourea, shake occasionally. This reagent should be freshly prepared. It should be checked for the presence of active reducing agent. To do this, place 2 ml of 1% $HgCl_2$ in a test tube and add the reagent drop wise. The addition of 3–5 drops will produce a copious precipitate of $HgCl_2$ if adequate thiourea is present.
5. *85% sulphuric acid*: Add 85 ml of sulphuric acid to 15 ml distilled water slowly.
6. *Standard*: 1 mg of ascorbic acid in 10 ml of 4% TCA. Take 1 ml from this and dilute to 6 ml.

12.6.3. Procedure

1. To 1 ml of pooled seminal plasma, add 6 ml of 6% TCA. Stir thoroughly.
2. Centrifuge to get a clear supernatant.
3. Add 300 mg of acid washed Norit to the supernatant. Shake vigorously and filter.
4. To 2 ml of filtrate, add 0.5 ml of dinitrophenyl hydrazine.
5. Incubate the tubes for 3 hours at 37°C after stoppering them. Cool the tubes by keeping them in the ice water bath.
6. Add 2.5 ml of 85% sulphuric acid drop by drop to avoid sudden rise in temperature. Shake the tubes thoroughly.
7. After half an hour bring the tubes to room temperature.

8. Record the absorbancy of the colour developed at 540 nm.
9. Control : For each sample use 2 ml of filtrate without dinitrophenyl hydrazine.

12.6.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu\text{g of standard} = \frac{\mu\text{g of ascorbic acid in ml of seminal plasma.}}$$

12.7. SEPARATION OF FREE AMINOACIDS USING TWO-DIMENSIONAL DESCENDING PAPER CHROMATOGRAPHY

12.7.1. Principle

The stationary phase acts as adsorptive force which binds with substances by hydrogen bonding or Vander waals force or ionic exchange. Non polar solvent in the mobile phase acts as a driving force and partitioning force.

12.7.2. Reagents

1. 80% ethanol :
 2. *Solvent I* : Prepare by mixing butanol 120 ml ; acetic acid 30 ml and distilled water 50 ml.
 3. *Solvent II* : Dissolve 160 gm of phenol in 40 ml of distilled water and to this add 1 ml of ammonia.
- 0.2% *ninhydrin solution*: Dissolve 200 mg of ninhydrin in 100 ml of acetone.

12.7.3. Procedure

1. Add 1 ml of 80% ethanol to 0.3 ml of seminal plasma.
2. Centrifuge and separate the supernatant. Again add 0.5 ml of 80% ethanol to the supernatant in order to check whether the supernatant is free from protein.
3. Centrifuge again to see whether there is any precipitate.
4. Add 3 volumes of chloroform to the clear supernatant.
5. Pipette out the aqueous phase alone into a cleaned dried small vials.

6. Spot the sample following the method of Smith (1968) on Whatmann No. 1 chromatographic paper and allow to run in the solvent system I, for the first direction and for the second direction use solvent system II.
7. Dry the paper and dip it in the 0.2% ninhydrin solution to locate the spots.
8. Again dry the paper in air and in oven at 105°C for 10—15 minutes.
9. Mark all the spots. Determine the Rf value and identify the spots by comparing with authentic standards.

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**ELECTROPHORETIC SEPARATION OF PROTEIN
FRACTIONS OF SEMINAL SUBSTANCES OF
SCYLLA SERRATA ***

13.1. INTRODUCTION

Among crustaceans, the cirripede seminal plasma has been reported to contain a large quantity of proteins (Barnes and Blackstock, 1974). In *S. serrata* too, the seminal plasma is proteinaceous (Uma, 1982). Since a copious quantity of seminal plasma is transferred to the female reproductive tract and stored in the spermatheca for a long time, the seminal plasma is suggested to act as a nutrient medium for sperm maintenance. The present experiment is designed to verify this supposition by electrophoretically separating the proteins of seminal plasma stored in the vas deferens as well as in the spermatheca of the mated females.

**13.2 METHOD OF COLLECTION OF SEMINAL PLASMA AND
SPERMATOPHORES**

(Vide Expt. No. 12)

13.2.1. Sample preparation

Seminal plasma : Dilute 0.1 ml of seminal plasma with 1 ml of 40% sucrose and from this take 0.2 ml of sample per one gel tube.

Spermatophores : Homogenise 100 mg of spermatophores with 1 ml of 40 % sucrose, centrifuge and take 0.2 ml of supernatant.

* Prepared and verified by K. Uma, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

13.3. COLLECTION OF SPERMATHECAL FLUID

In *S. serrata*, the prepubertal females having a white thread like ovary is always unmated, which is evidenced from the absence of spermatophores in the spermathecal smear. However when the ovary matures to stage II, spermathecal smear is rich in spermatophores. Mated *S. serrata* is available from stage II to IV of ovarian maturation (Ezhilarasi, 1978 ; Ezhilarasi and Subramoniam, 1982).

13.3.1. Method of collection of spermathecal fluid from virgin crabs

Remove the entire spermatheca from the virgin female (stage II—ovary) ; wash the spermatheca with distilled water to remove the adhering haemolymph.

Transfer it into a clean watch glass ; puncture the spermatheca and collect the contents.

Dilute 0.2 ml of this with 1 ml of 40% sucrose. From this take 0.2 ml for one gel tube.

13.3.2. Method of collection of spermathecal fluid from the mated female crabs

Remove the spermatheca from the mated females (Stage II—ovary) ; wash the spermatheca with distilled water, transfer it into a clean watch glass. Puncture it ; collect the contents, centrifuge at 2000 rpm for 5 minutes to separate the fluid free from the spermatophores.

Dilute 0.2 ml of supernatant (spermathecal fluid) with 1 ml of 40% sucrose and take 1.2 ml for one gel tube.

Homogenise 150 mg of the spermatophores taken from the mated spermatheca with 2 ml of 40% sucrose, centrifuge, take 0.2 ml of the supernatant for one gel tube.

For electrophoretic method (Davis, 1964) *vide* Expt. No. 3.

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V. NEUROENDOCRINE CONTROL OF REPRODUCTION

STAINING METHODS FOR NEUROSECRETORY SYSTEM IN CRUSTACEANS*

14.1. INTRODUCTION

A neurosecretory system consists of neurosecretory cells (NSCs) and a neurohaemal organ for synthesis and storage of neurohormones respectively. In the central nervous system (CNS) the distribution of NCSs is confined to the brain, thoracic ganglia, circum-oesophageal connective and eyestalk. The NSCs elaborate secretory materials which can be demonstrated by staining techniques (Gomori, 1939, 1941, 1950; Dogra and Tandan, 1964). Among them, Gomori's (1939) chrome-hematoxylin phloxine (CHP) and Gomori's (1950) paraldehyde fuchsin (PF) are the most common.

14.2. PRINCIPLE

Chrome-hematoxylin, basic fuchsin and Victoria blue are the commonly used basic dyes for staining the NSCs. The rationale of chrome-hematoxylin phloxine and paraldehyde fuchsin staining of the neurosecretory material (NSM) is based on the affinity of these stains for the acidic groups, appearing after oxidation of NSM with oxidizing agents such as performic acid and potassium permanganate. Raabe (1980) thus found an increased neurosecretory activity after increasing the time of oxidation for the NSCs in the pars intercerebralis of insects. The specific oxidation of the NSM involves the formation of cysteic acid from both cysteine and cystine which are present in enormous quantity in the NSM of insects (Dogra and Tandan, 1964) and crustaceans (Lake, 1970). The same oxidation process also produces free aldehyde groups, to which the basic stains can bind (Gabe, 1953).

* Prepared and verified by M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

14.3. MATERIAL

Brain and thoracic ganglion of the anomuran crab, *Albunea symnista*

14.4. Preparation of Tissue

1. Dissect the animal in a medium of 0.9% saline solution.
2. Remove the brain, thoracic ganglion and wash well with the saline.
3. Fix it in Bouin's fixative for 18—24 hours.
4. Dehydrate in alcohol, clear in xylol and embed in paraffin wax (m.p. 54°C—56°C).
5. Cut sections at 6 μ m thickness.

14.5. GOMORI'S (1939) CHROME-HEMATOXYLIN PHLOXINE METHOD [BARGMANN'S (1949) MODIFICATION]

14.5.1. Reagents

1. *Bouin's fluid*: Vide expt. No. 1.
2. *Mordant solution*: Dissolve 4 gm of chromium potassium sulphate in 100 ml of Bouin's fluid by heating gently to about 50°C.
3. *Oxidizing solution*: Add 10 ml of 2.5% potassium permanganate (2.5 gm in 100 ml distilled water) to 10 ml of 5% sulphuric acid (5 ml in 95 ml distilled water). To this add 80 ml of distilled water.
4. *Chrome-hematoxylin*: Add 50 ml of 1% hematoxylin (1 gm in 100 ml distilled water) to 50 ml of 3% chromium ammonium sulphate (3 gm in 100 ml distilled water). To this add 2 ml of 5% potassium dichromate (5 gm in 100 ml distilled water) and 1 ml of 5% sulphuric acid.
5. *1% Oxalic acid*: Add 1 ml of oxalic acid to 99 ml of distilled water.
6. *0.5% Acid alcohol*: Add 0.5 ml of hydrochloric acid to 99.5 ml of 70% alcohol.

7. 0.5% *Phloxine*: Dissolve 0.5 gm of phloxine in 100 ml of distilled water.
8. 5% *Phosphotungstic acid*: Dissolve 5 gm of phosphotungstic acid in 100 ml of distilled water.

14.5.2. Procedure

1. Bring sections to distilled water.
2. Immerse the sections in mordant solution for 12—24 hours at 37°C (mordant forms a link between the tissue and the stain).
3. Wash in running tap water until the sections become colourless.
4. Keep the sections in oxidizing solution for 5 minutes.
5. Bleach in 1% oxalic acid for 1 minute.
6. Wash in running tap water for 5 minutes.
7. Rinse in glass distilled water.
8. Stain in chrome-hematoxylin for 30—40 minutes at 4°C.
9. Differentiate in 0.5% acid alcohol for 30 seconds.
10. Rinse in running tap water for 2 minutes.
11. Stain in 0.5% phloxine for 2—3 minutes.
12. Transfer to 5% phosphotungstic acid for 5 minutes.
13. Wash in running tap water for 5 minutes.
14. Differentiate in 70% alcohol.
15. Dehydrate, clear in xylol and mount in DPX.

14.5.3. Result

Neurosecretory substance—deep purple.

Nuclei—purple. Background—pinkish red.

14.6. GOMORI'S (1950) PARALDEHYDE FUCHSIN METHOD [CAMERON AND STEELE'S (1959) MODIFICATION]

14.6.1. Reagents

1. *Gomori's fluid*: Dissolve 15 gm of potassium permanganate in 50 ml of distilled water. Add to this 0.1 ml of concentrated sulphuric acid.

2. *2.5% Sodium bisulphite* : Dissolve 2.5 gm of sodium bisulphite in 100 ml of distilled water.
3. *Paraldehyde fuchsin* : Add 1 gm of basic fuchsin to 200 ml of boiling distilled water. Boil once again for 1 minute, cool and filter. Add 2 ml each of concentrated hydrochloric acid and paraldehyde solution (100%) to the filtrate. Close the container air tight and leave it at room temperature. Wait until the maximum precipitation of fuchsin occurs at the bottom of the bottle. At this time, the solution will lose its reddish fuchsin colour. Filter the solution and discard the filtrate. Dry the precipitate on the filter paper (Temperature 57°C—59°C is suitable for this). Remove the dry crystals from the filter paper and store in a reagent bottle. Dissolve 0.25 gm of the crystal in 50 ml of 70% alcohol. The solution can be used upto 6 months.
4. *Halmi's mixture* : Dissolve 0.2 gm of light green and 1.0 gm of orange G in 100 ml of distilled water. Shake well. To this add 0.5 gm of phosphotungstic acid and 1 ml of glacial acetic acid. The stain need not be filtered.
5. *0.2% Acetic acid* : Add 0.2 ml of acetic acid to 99.8 ml of distilled water.

14.6.2. Procedure

1. Bring sections to distilled water.
2. Oxidise in Gomori's fluid for 1 minute.
3. Rinse in 2.5% sodium bisulphite solution until all permanganate stain is removed (a few seconds).
4. Rinse in distilled water.
5. Transfer the slide through 30% to 70% alcohol.
6. Stain in the paraldehyde fuchsin solution for 2—10 minutes.
7. Quickly wipe the back of the slide and rinse in 95% alcohol.

8. Transfer the slide to a second bath of 95% alcohol for 1—5 minutes (until no more paraldehyde fuchsin comes away).
9. Bring to water through 70% and 30% alcohols.
10. Counter stain in Halmi's mixture for 20—30 seconds.
11. Wipe the back of the slide and differentiate in 95% alcohol containing 0.2% acetic acid until no more stain comes away (2—3 minutes).
12. Rinse in 95% alcohol.
13. Dip the slides in absolute alcohol (2 changes), clear in xylol and mount in DPX.

14.6.3. Results

Neurosecretory cells—deep purple, dark blue and pale violet.

14.7. IDENTIFICATION OF NEUROSECRETORY CELLS AND THEIR PATHWAYS IN INTACT NEUROENDOCRINE ORGANS

14.7.1. Introduction

In crustaceans, a large number of NSCs are distributed on the surface of the brain, thoracic ganglia and optic lobe. The NSCs as well as their pathways are identified by using performic acid/Victoria blue staining method of F.D. Humberstone, modified by Dogra and Tandan (1964). This method was originally used to study the axonal pathways of the NSCs in the pars intercerebralis of insects.

14.7.2. Materials

Brain, thoracic ganglion and eyestalk of the penaeid prawn *Metapenaeus monoceros*.

14.7.3. Performic Acid/Victoria Blue Method of F.D. Humberstone [Dogra and Tandan's (1964) modification]

14.7.3.1. REAGENTS

1. *Performic acid*: Add 4 ml of 30% hydrogen peroxide (30 ml in 70 ml distilled water) and 0.5 ml of concentrated sulphuric acid to 40 ml of 98% formic acid (98 ml in

2 ml distilled water). Allow the mixture to stand for at least an hour. Use preferably within 24 hours (use 100 vol. hydrogen peroxide, not more than 3 weeks after opening the bottle).

2. *Victoria blue solution*: Dissolve 0.5 gm of dextrine, 2 gm of Victoria blue RN₂₇₅ and 4 gm of resorcin in 200 ml of distilled water. Heat the mixture to boil. While boiling briskly add 25 ml of boiling 29% ferric chloride. Boil for 3 minutes and cool. A heavy precipitate forms. Filter and dry the precipitate in an oven at 50°C. Dissolve the precipitate in 400 ml of 70% alcohol. Shake the mixture well. To this add 4 ml of concentrated hydrochloric acid and 6 gm of phenol. Use the solution after 2 weeks for better results. The stain will last for months.

14.7.3.2. PROCEDURE

1. Dissect the animal in a medium of 0.9% saline solution to expose the neuroendocrine organs.
2. Fix *in situ* using 10% formaldehyde saline (Add 9 ml of 0.9% physiological saline to 10 ml of formaldehyde) for 1—2 minutes.
3. Remove the neuroendocrine organs, wash well with the fresh fixative and keep it in the fixative for 24 hours.
4. Wash well in tap water for 2 or 3 hours.
5. Rinse in distilled water 10—20 minutes.
6. Remove the water on the surface of the tissue with filter paper.
7. Oxidize the tissue with performic acid until it becomes transparent (5 minutes or more).
8. Wipe off the excess performic acid in the tissue with filter paper.
9. Wash thoroughly in distilled water for 20—30 minutes.
10. Dehydrate in 30% and 70% alcohols.
11. Stain the organs in Victoria blue solution for 12—18 hours (duration of staining depends on size of the organ).
12. Quickly blot off excess stain with filter paper.

13. Differentiate in 70% alcohol (3 or 4 changes until the excess stain is removed).
14. Dehydrate in 90% and absolute alcohols; clear in cedar-wood oil for 2—4 hours.
15. Rinse in xylene for 2—5 minutes to remove the cedar wood oil.
16. Mount in DPX.

14.7.3.3. RESULTS

Cell body—blue or greenish blue; Proximal portion of axons—greenish blue; Neurosecretory pathway—light greenish blue; Storage and release organ—blue or dark blue; Background—unstained or faint blue.

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A CLASSIFICATION OF NEUROSECRETORY CELLS OF CRUSTACEA*

15.1. INTRODUCTION

In Crustacea, the distribution of NSCs is limited to the brain, thoracic ganglia, circum-oesophageal connectives and eyestalk. Enami (1951) first described different types of NSCs and mapped them in the brachyuran crab, *Sesarma dehaani*. Following this, there were numerous reports on the classification of NSCs in a variety of crustaceans belonging especially to the superior orders (Durand, 1956 ; Matsumoto, 1958 ; Adiyodi and Adiyodi, 1970). One of the compelling inference from these studies is that the morphology of the NSCs in different forms is highly variable and species-specific. In the present experiment a classification of NSCs of an anomuran crab is attempted employing various criteria such as size, shape, location, tinctorial properties and cytological details.

15.2. MATERIAL

Brain and thoracic ganglion of the anomuran crab, *Albunea symnista*.

15.3. PROCEDURE

1. Observe the slides prepared in the Expt. No. 14.
2. Note the size, shape, location and other cytological details of the NSCs.
3. Classify the NSCs into different types based on the characteristics mentioned in the step 2.
4. Draw the camera lucida drawings of NSCs (Fig. 1).

* Prepared and verified by M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

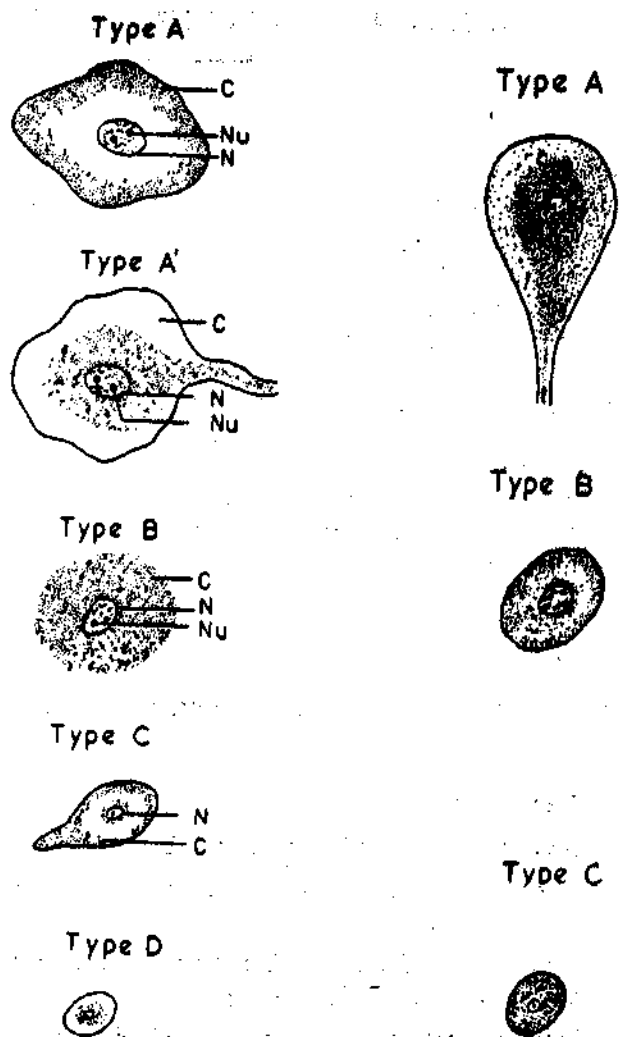


Fig 1.
Types of NSCs in the brain (a) and thoracic ganglion (b) of *A. symmista*.

5. Map the distribution of NSCs in different regions of the brain and thoracic ganglion. For this, first draw the outline of intact neuroendocrine centres. By using various symbols, indicate the location of individual NSCs as shown in Figs. 2 and 3.

15.4. OBSERVATIONS

15.4.1. NSC types of the brain (Fig. 1a)

A type NSC: These NSCs are 45 μm -50 μm in diameter. The nucleus is oval in outline and possesses two nucleoli. The cytoplasm is flaky due to the accumulation of secretory granules. These cells are distributed in the dorsal and ventral median regions of the protocerebrum. The axons are indistinguishable.

A' type NSC: It is similar to the A type NSC, except for the presence of an axon. Cells of this type are mainly located along with the A type in the dorsolateral and ventromedian regions of the protocerebrum.

B type NSC: These cells are oval and 35 μm -40 μm in diameter. The nucleus is prominent, but without a distinct nucleolus. These cells are known to be distributed in the anterolateral region of the protocerebrum with a few cells scattered in the ventrolateral region. As in the A type, axons are wanting.

C type NSC: These are conical or club shaped, and are few in number, measuring 25 μm - 30 μm in diameter. They are distributed in the dorsal and ventral regions of the deutocerebrum. A few cells are also seen near the A type.

D type NSC: This type is spherical without an axon, and measures 8 μm -10 μm in diameter. The cytoplasm is scanty, but possesses dark blue granule. Cells of this type are confined exclusively to the dorsal region of the protocerebrum.

15.4.2. NSC type of the thoracic ganglion (Fig. 1b)

A type NSC: It is monopolar, uninucleolated and 25 μm -30 μm in diameter. The shape of the nucleus varies from flat to crescent. These cells are located in the dorsal and ventral regions of the neuropile.

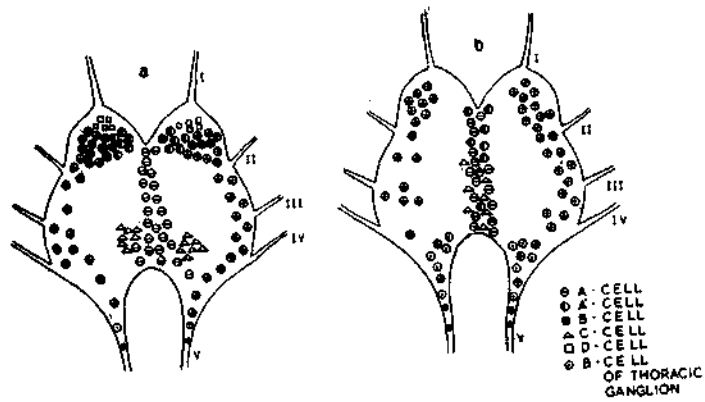


Fig. 2.

Distribution of NSCs in the brain of *A. symnista*. (a) dorsal side, (b) ventral side. I—optic nerve, II—Antennulary nerve, III—Antennary nerve, IV—Maxillary nerve, V—Circum-oesophageal connective.

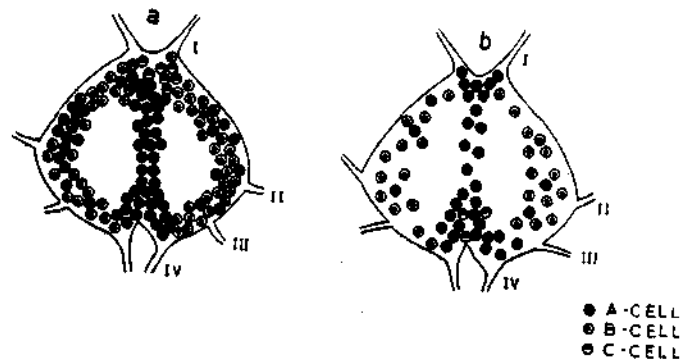


Fig. 3.

Distribution of NSCs in the thoracic ganglion of *A. symnista*. (a) dorsal side, (b) ventral side. I and IV—Thoracic nerves, III and IV—Nerves to thoracic appendages.

Y-ORGANECTOMY IN THE CRAB, *SCYLLA SERRATA**

16.1. INTRODUCTION

Gabe (1953, 1956) first described the Y-organ in 58 species of Malacostraca. The Y-organ is a glandular structure of epidermal origin. It is located in the antennary/maxillary segments. The Y-organ varies in gross morphology and location in different species of crustaceans (Spindler *et al.*, 1980). It is conical in the Brachyura, tentacular in the Natantia and foliaceous in Isopoda. In position, appearance and function, the Y-organ is similar to the prothoracic gland of insects. In Crustacea, there are two types of Y-organ. They are (1) Y-organs isolated from the hypodermis (brachyurans) and (2) Y-organs connected to or even integrated into the hypodermis (macrurans).

Bilateral removal of the Y-organs prevents molting in crustaceans and reimplantation leads to the resumption of the normal molting process (Echalier, 1954, 1955, 1959; Passano and Jyssum, 1963; Burghause, 1975). The molt inhibiting hormone (MIH) from the X-organ/sinus gland complex controls the synthesis and secretion of molting hormone (ecdysone) from the Y-organ (Kleinholz and Keller, 1979; Spindler *et al.*, 1980).

In recent years, the importance of the ecdysial glands of Crustacea namely the Y-organ has been realised in the reproductive process especially the multiplication of oogonial cells and the stimulation of vitellogenin synthesis in the extra-ovarian sites (Arvy *et al.*, 1956; Demeusy, 1962).

* Prepared and verified by M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

In this experiment the method of Y-organ ablation closely follows the technique of Echaliér (1959).

16.2. MATERIAL

Alive crab, *Scylla serrata*.

16.3. REAGENTS

Methylene blue solution : Dissolve 0.2 gm of methylene blue in 100 ml of distilled water. Add 10 ml of the above solution to 90 ml of 0.9% physiological saline (0.9 gm sodium chloride in 100 ml distilled water).

16.4. PROCEDURE

1. Hold the specimen immobile on a dissection board with the face uppermost under a dissection microscope.
2. Place the dissection board in a tray containing 0.9% physiological saline.
3. Cut a 2.5 mm square piece of the pterygostomian region of the exoskeleton, bordering on the ventral edge of the sub-orbital region and directly below the cornea of the stalked eye by means of a dental drill.
4. Incise the epidermis on three edges of the hole and lift back carefully, revealing the Y-organ which is attached to the epidermis (The Y-organ has a yellowish appearance against the bluish white colour of the surrounding muscle and connective tissue).
5. Remove the Y-organ with sharpened watch maker tweezers.
6. Replace the epidermis in the original position and seal with molten paraffin wax.
7. Keep the organ in a cavity block with the physiological saline containing one drop of methylene blue solution.
8. Observe the gland under low magnification.

16.5. RESULTS

The cells of the Y-organ with secretory products stain deep blue.

16.6. REFERENCES

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**EFFECT OF EYESTALK ABLATION ON
THE OVARIAN MATURATION OF AN OCYPOD CRAB,
OCYPODA MACROCERA USING WINDOW METHOD***

17.1. INTRODUCTION

X-organ/sinus gland complex resident in the eyestalk of decapod crustaceans contains the inhibitory hormones for molting and reproduction (MIH, GIH) (Adiyodi and Adiyodi, 1970). Therefore, the eyestalk extirpation results in the removal of the inhibitory factors both for molting and reproduction. The removal of MIH or GIH perforce induces the activity of either MH or GSH, thus bringing about precocious molting or reproduction (Adiyodi and Adiyodi, 1970). The present experiment is designed to find out the effect of eyestalk ablation in the females in early stages of vitellogenesis using window method. This method was introduced by Gomez and Nayar (1965) for observing directly the ovarian maturation when the animals are subjected to eyestalk ablation studies. This method is especially useful when the population is heterogenous and breeds continuously.

17.2. PROCEDURE

1. For experimentation, select two sets of crabs at early stage of vitellogenesis using window method.
2. Make a small square hole at the dorsal side of the carapace (cephalothorax region) just below the eyestalk with the help of sterilized scissors and forceps.
3. Immediately after exposure, assess the ovarian stage by directly observing the color and select the early vitellogenic (vitellogenesis-I) crab (*vide* Expt. No. 1.).

* Prepared and verified by K. Nadarajalingam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

4. Immediately after operation, wipe off the hemolymph with cotton and apply terramycin ointment to avoid infection.
5. Place a little quantity of cold paraffin wax over the wound along with cotton.
6. Label the crab on the dorsal side of the carapace with paint.
7. After 5 minutes take one set of these ovarian-stage assessed crabs for eyestalk ablation and the other set is used as control.
8. Cut the eyes at the basal region of the non-retinal portion with sterilized scissors and apply cold molten wax in the cut portions to avoid the loss of hemolymph.
9. Place the crab in the empty tank for sometime to avoid the animal immediately approaching the water and sand.
10. For control crabs, after assessing the stage by window method, make a small cut in the tip of the pleopod and heat-cauterize the cut end.
11. Place the destalked and control crabs in separate tanks.
12. Maintain the same field salinity in the laboratory for control and experimental crabs.

17.3. OBSERVATION

1. Compare the ovarian stages of control and experimental crabs at 5 days interval.
2. Note the cytological and cytophysiological changes in the ovary and other endocrine centres with an interval of 5 days.

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EYESTALK LIGATION EXPERIMENTS ON THE FAIRY SHRIMP *STREPTOCEPHALUS DICHOTOMUS**

18.1. INTRODUCTION

The endocrine system of lower crustaceans such as anostracan also shows a similarity with that of decapod crustaceans, especially in the possession of a stalked eye in which is present the X-organ sinus gland complex (Lake, 1969). In the fairy shrimp *Streptocephalus dichotomus*, the sinus gland is located inbetween the lamina ganglionaris and the optic medulla. The cells in this gland show variation in their secretory contents during reproductively quiescent and reproductively active periods (Munuswamy, 1982). The effect of eyestalk ligation on vitellogenesis depends on the phase of the annual reproductive cycle (Weitzman, 1964). The present experiment is designed to study the effect of eyestalk ligation on ovaries and clutch formation, during different phases of the female reproductive cycle of the fairy shrimp *S. dichotomus*.

18.2. MATERIALS

Specimens of *S. dichotomus* (males and females).

18.3. PROCEDURE

1. Select 3 sets of fairy shrimps of different maturity.
Group I : immature stage (10-12mm)
Group II : mature stage (15-25 mm)
Group III : spent stage (15-25 mm)
2. Prechill the animals for a few seconds in ice.

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3. Ligate the eyestalks of the above three groups of fairy shrimps using human hairs ; loope around the base of the eyestalk.
4. Maintain experimental as well as control females (without ligation), in separate finger bowls with pond water.
5. Perform all the experiments under normal day light condition.
6. Do not feed the fairy shrimps during the experiments.

18.4. OBSERVATION

Observe the nature of ovary, shell glands, ovisac and frequency of clutch formation in the three groups of fairy shrimps. Compare the results with that of control groups.

18.5. INFERENCE

As the reproductive cycle of the fairy shrimp *S. dichotomus* is short (48 hours), the efficacy of the ligation could be evaluated within 6 to 15 hours after ligation. Direct observation on the changes occurring in the appearance of eggs in the lateral part of the oviduct, eggs in the ovisac and the secretory nature of the shell gland could be made through the transparent body wall.

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**EFFECT OF ALTERED PHOTOPERIODISM
(ALTERED INTENSITY AND DAY LENGTH)
ON NEUROENDOCRINE FUNCTION
AND OVARIAN MATURATION IN AN
OCYPOD CRAB, *OCYPODA MACROCERA****

19.1. INTRODUCTION

In marine invertebrates, light forms a significant external signal ('Zeitgeber', Aschoff, 1960) to control various reproductive activities (Segal, 1970). The initiation and synchronization of spawning, gonad maturation and sex determination are light-dependent reproductive activities and appear to be mediated through photo-neuroendocrine pathways (Scharrer, 1964). In *Porcellio dilatatus*, the extension of photoperiod lengthens the period of reproduction and delays the molt (Mocquard *et al.*, 1976 a, b). In decapod crustaceans such as *Pachygrapsus marmoratus* (Pradeille-Rouquette, 1976) and *Palaemonetes pugio* (Little, 1968) the administration of longer photoperiod accelerates reproduction. The above mentioned examples clearly suggest the role of day length on the initiation of reproductive processes in lower as well as higher crustaceans.

However, the specific role of wavelength and light intensity on reproductive processes has received only meagre attention. In sea pen, *Cavernularia obesa*, the intensity of illumination determines the time course for gamete release (Mori, 1960). More secretory activity is noticed in MTGX1 (Medulla terminalis ganglionaris X-organ) and MTGX2 of eyestalk in *Palaemon serratus* when subjected to light-dark experiments (Van Herp *et al.*, 1977). Likewise, in crayfish *Orconectes clypeatus*, Finger mann and Oguro (1962) found the stimulation of neurosecretory

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cells in medulla externa of eyestalk when administered to continuous light. So it is evident that the photoperiod regulates the molting and reproduction through neuroendocrine pathways.

19.2. PRINCIPLE

Artificial illumination is effected through the neuroendocrine pathways and regulates the process of molting and reproduction by altering the neuroendocrine secretion in endocrine centres. The light regime suppresses the MIH factor in the eyestalk and stimulates the MH when light is administered at the time of molt. During the intermolt period the inhibition of MIH factor stimulates the production of GSH in the brain and TG of females and androgenic gland hormone in males.

19.3. PHOTOPERIODISM CHAMBER

The square photoperiodism chamber is made with wood at the size of 193L × 140 W × 148 H (in cms) and is covered by black cloth. At the corners of upper side of the chamber, inverted 'U' shaped tubes are fixed for free aeration. Three 4300 K fluorescent white tube lights are fixed inside the chamber. The intensity of light is altered by changing the height of the light and the same is measured with EEL Lux meter.

19.4. ALTERED INTENSITY AND CONSTANT DAYLENGTH

19.4.1. Procedure

1. Prior to experiment, assess the ovarian stages of all crabs by window method (*vide* Expt. No. 17).
2. Select the following four sets of crabs at early stage of vitellogenesis (vitellogenesis-I).

Experimental

- I. With intact eyestalk.
- II. With painted (black paint) eyestalk.

Control

- I. With intact eyestalk.
- II. With painted eyestalk.

3. Keep the experimental sets inside the photoperiodism chamber (4 crabs/tank) and the control sets outside the chamber.
4. Maintain the same field salinity and simulate the environmental conditions in all the tanks.
5. Use different light intensities such as 140, 300, 450, 640, 800 Lux by altering the height of the fluorescent tube light.
6. At a time, take the single light intensity along with fixed daylength (12L : 12D) to study the immediate effect on the ovarian maturation. Likewise, alter the intensity five times (five intensities) with constant daylength (12L : 12D).

19.4.2. Observation

1. Compare the ovarian stages of control and experimental crabs.
2. With a 5 day interval observe the cytological and cytophysiological changes of ovary and the neuroendocrine centres.

19.5. FIXED INTENSITY AND ALTERED DAYLENGTH

19.5.1. Procedure

1. Repeat the steps 1-4 of the previous experiment (19.4).
2. Use the suitable intensity which is standardized in the first experiment (19.4).
3. Alter the daylength as OL : 24D ; 4L : 20D ; 8L : 16D ; 12L : 12D ; 16L : 8D ; 20L : 4D and 24L : 0D.
4. At the time of experiment, use the fixed intensity and select a single daylength among the seven daylengths mentioned above. Likewise, conduct seven experiments with seven daylengths with fixed intensity and find out the suitable daylength, favourable for the ovarian maturation.

19.5.2. Observation

- (i) Make a comparison between the ovarian stages of control and experimental crabs.

- (ii) Observe the cytological and cytophysiological changes of ovarian maturation and the neuroendocrine centres with the interval of five days.

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VI. REPRODUCTIVE ECOLOGY

**DETERMINATION OF REPRODUCTIVE
PERIODICITY IN THE INTERTIDAL MOLE
CRAB *EMERITA ASIATICA****

20.1. INTRODUCTION

Food supply may be regarded as the primary factor controlling growth rate and egg production in the natural populations of Crustacea (Wenner *et al.*, 1974). The faster growth rate can also lead to an altered age/size at sexual maturity. Thus environmental factors not only influence the percentage of berried female in a population, but also the size of sexual maturity. Therefore, in determining the reproductive cycle of any population of Crustacea, the minimum size at sexual maturity must be considered first. Among the various methods employed to determine the reproductive activity of marine invertebrates (Giese, 1959), by far the gonad index is the most common. This is calculated as the ratio of the gonad wet weight (or volume) to the wet weight (or volume) of the whole animal, expressed as a percentage (Giese and Pearse, 1974). Since most of the decapod crustaceans carry the eggs in the pleopods till hatching, the percentage occurrence of ovigerous females in a population can also be used as an index of reproductive activity. Again, the rate of egg development also varies according to the sea water temperature and hence an assessment of the mean egg developmental rate is also necessary for correctly assessing the reproductive activity. In this experiment various methods as applied to estimating the female reproductive cycle of an anomuran crab *Emerita asiatica* are described.

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20.2. METHODS

20.2.1. Determination of stage at sexual maturity

Collect female crabs of different sizes and measure the carapace length (CL).

Find out the lowest size class of the ovigerous females in a population. This should be determined for all the samples collected throughout the year.

Determine arbitrarily the size range at which the females begin to lay eggs (In *Emerita* 19 to 22 mm CL). For gonad index and other methods, consider only females above this size range.

20.2.2. Incidence of ovigerous female

Find out the percentage of ovigerous females in the samples collected in different months of the year. Plot the percentage of ovigerous females against time.

20.2.3. Egg mass index

Wash the crab to remove the adhering sand particles and weigh in a physical balance.

Remove the pleopods along with the attached eggs and weigh after blotting dry.

Strip off the eggs from the pleopodal hairs and determine the weight of the pleopod. The weight of the eggs can be calculated by subtracting the weight of the pleopods from the initial weight of the pleopods with attached eggs.

Calculate the egg mass index using the formula :

$$\text{Egg mass index} = \frac{\text{Weight of the egg}}{\text{Weight of the body}} \times 100$$

20.2.4. Mean egg developmental stages (MEDS)

To find out the mean egg developmental stages, determine the different stages in the egg development occurring in the pleopod using the key given in Table 1.

Classify the eggs of the collection sample into one of the ten developmental stages (Table 1). Suppose we have a sample

TABLE 1. Classification of Egg Development in *Emerita asiatica*

Stage	Description
I	Yellow yolk granules seen ; egg mass bright orange in colour.
II	Cleavage has taken place and blastomeres are seen ; egg mass bright orange in colour.
III	A yolk-free white streak makes its appearance in the animal pole ;
IV	One quarter of the yolk cleared ; the white band encircles the yolk material which is now in the centre ; at the animal pole a periodic twitching is recognized ; red pigment spots are seen at the edge of the yolk ; colour of the egg mass is dull orange.
V	One third of the yolk cleared ; two eye spots appear ; red spots prominent and seen at the end of the animal pole ; colour of the egg mass dull orange.
VI	Egg mass brownish orange in colour ; eyes well developed ; yolk is found in the vegetal pole ; two thirds of the yolk cleared ; red pigments seen all over the white space.
VII	Egg mass greyish orange in colour ; yellow yolk is found as two clusters in the centre ; appendages of the embryo are developed ; heart beat seen ; eye spots very well developed.
VIII	Egg mass pale grey in colour ; colourless yolk in the form of oil globules seen just below the eyes as two pockets ; heart beat more prominent ; embryo almost completely developed.
I X	Embryo fully formed ; egg mass white in colour ; no yolk globules seen ; about to be released.
X	Released zoea larvae.

Adapted from Subramoniam (1979).

collection of ten animals in January, 3 of them falling into 3rd stage, 2 of them falling into 5th stage and 5 of them falling into 8th stage, then the frequency distribution for the 10 animals will be represented as follows :

Month	No. of animals	Developmental stages										MEDS± Sd
		1	2	3	4	5	6	7	8	9	10	
Jan.	10	-	-	3	-	2	-	-	5	-	-	

Compute the MEDS using the formula

$$\text{MEDS} = (\sum Si \times fi) / \sum fi$$

where Si = stage 1, 2, 3,, 10 and

fi = the number of animals falling in the i^{th} developmental stages.

\sum = stands for the summation for all 10 stages.

From the above example

$$\text{MED} = \sum (Si \times fi) / (\sum fi)$$

$$= 59/10$$

$$= 5.9$$

Standard deviation (Sd) can be computed using the formula

$$Sd = \frac{(\sum Si^2 \times fi) - (\sum Si \times fi)^2 / (\sum fi)}{(\sum fi - 1)}$$

Find out the MEDS for all the 12 months in a year.

20.2.5. Gonad and hepatic indices

Dissect the crab and drain away the blood completely.

Carefully remove the ovary and hepatopancreas and transfer them separately to previously weighed cellophane paper without blotting. Blotting the tissue with the filter paper will result in the mature ovary containing fully ripe oocytes sticking to the paper.

Weigh the ovary and hepatopancreas in a monopan balance to the nearest 0.1 mg.

Calculate the gonad as well as hepatopancreatic indices by expressing their respective weights as a percentage of total

weight of the animal. For the ovigerous female the weight of the animal excludes the weight of the pleopodal eggs.

20.3. OBSERVATION

Plot the values of the egg mass index, MEDS, gonad and hepatic indices in the same graph and delineate the breeding intensities for atleast three consecutive years.

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DETERMINATION OF REPRODUCTIVE ACTIVITY IN SEA URCHINS*

21.1. INTRODUCTION

The most widely used quantitative method for assessing the reproductive activity is the gonad index (Giese and Pearse, 1974). But, in species such as sea urchins possessing considerable quantities of nutritive tissues in the gonad, both an increase and a decrease in gonad index may be a consequence of changes in the number of nutritive cells without a corresponding change in gametogenic cells (Moore, 1937 ; Pearse, 1969). Moreover, in such forms gametogenesis may occur at the expense of nutritive tissue, without a corresponding change in gonad index (Pearse, 1965). Hence, an alternate method for estimating the reproductive activity is given here. By this method, histological sections of the ovary are employed to follow the gonadal changes during reproductive cycle. This may be accomplished by the following two methods :

- i. By measuring the area occupied by gametogenic and nutritive cells (Holland and Holland, 1969)
- ii. Counting the different stages of oocytes and ova in the ovary (Vivek Raja, 1980)

21.2. MEASUREMENT OF THE AREA OCCUPIED BY DIFFERENT CELL POPULATIONS IN THE OVARY

21.2.1. Materials

Different stages of ovary of sea urchins.

21.2.2. Reagents

1. *Neutral buffered formaldehyde : Vide Expt. No. 2*

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt Arts College, Nandanam, Madras-600 035.

2. *Ehrlich's haematoxylin* : Dissolve 2 gm of haematoxylin in 100 ml alcohol. To this add 100 ml distilled water, 100 ml glycerine, 10 ml glacial acetic acid and 15 gm potassium alum. Keep it in sunlight for two months for natural ripening.
3. 1% *Acid alcohol* : Add 1 ml hydrochloric acid to 99 ml 70% alcohol.
4. 1% *Eosin—Y* : Dissolve 1 gm of eosin-Y in 100 ml of distilled water.

21.2.3. Procedure

1. Remove the ovary from the animal and fix it in neutral buffered formaldehyde.
2. Prepare paraffin sections at 7 μ m thickness and stain with haematoxylin and eosin-Y.
3. Keep the slide under microscope, which is fitted with a micrometer.
4. Measure the diameter of one ovarian lobe cross section using micrometer and then calculate the area using the formula: $A = \pi r^2$, where, A = area of the lobe, r = radius.
5. The image of the cross section should be projected by means of a camera lucida into a graph sheet.
6. Outline the regions occupied by each of the following populations.
 - (a) the nutritive phagocyte population.
 - (b) the gamete population (ova)
 - (c) the gametogenic cell population (oocytes)

These three populations comprise the entire measurable area of the cross section ; the areas occupied by the connective tissue muscular layer and the peritoneal layer of the gonad should be excluded.

7. Measure the area occupied by each cell population.

8. The cross sectional area (in square microns) of the cell population should be calculated by the following formula :

Area of cell population in square microns	=	Area of lobe cross section in square microns	=	Area of the cell population in graph in cm ² Total area of all three cell popula- tions in graph in cm ²
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21.2.4. Inference

The area occupied by the mature gametes (ova) in a section of ovary is directly proportional to the reproductive activity of the animal.

21.3. COUNTING THE FREQUENCY OF DIFFERENT STAGES OF OOCYTES AND OVA IN THE OVARY

21.3.1. Introduction

For the continuously breeding invertebrates such as the sea urchin, *Salmacis virgulata* the rate of reproductive activity can be studied by estimating the frequency of different stages of oocytes and ova in the ovarian sections.

21.3.2. Procedure

1. Prepare the histological sections of the ovary and stain them with haematoxylin and eosin-Y.
2. Observe the slide under microscope, measure the diameter of the oocytes and ova ; classify them into different size classes.

Note : Classify the oocytes and ova into different size classes as given below (Table 1). The oocytes measuring upto 10 μm

should be included in size class I. The size range from 10.1 μm to 130 μm should be divided into 12 size classes (Table 1).

TABLE 1

Oocytes and ova size classes	Oocytes and ova diameter in μm
I	Upto 10.0 μm
II	10.1—20.0
III	20.1—30.0
IV	30.1—40.0
V	40.1—50.0
VI	50.1—60.0
VII	60.1—70.0
VIII	70.1—80.0
IX	80.1—90.0
X	90.1—100.0
XI	100.1—110.0
XII	110.1—120.0
XIII	120.1—130.0

3. The diameter of the oocyte with nucleoli and ova with nuclei alone should be measured (Holland, 1967 ; Gonor, 1973 ; Vivek Raja, 1980).
4. The diameter of 200 such cells should be measured sequentially.
5. Record your data in table and draw a histogram. The frequency of the ova should be marked in the histogram by a specific colour. The oocytes belonging to size class VIII and above are called as terminal oocytes.

21.3.3. Inference

The percentage of ova and terminal oocytes present in the ovarian sections is directly proportional to the reproductive activity of the animal.

21.4. REFERENCES

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VII. FERTILIZATION AND EARLY DEVELOPMENT

INDUCED SPAWNING IN SEA URCHINS*

22.1. INTRODUCTION

The gonad wall of the sea urchin consists of an outer epithelium, the outer surface of which is bathed in the perivisceral fluid, a middle layer of conspicuous bands of smooth muscles and connective tissue and an inner layer of developing gametes with nutritive cells (Vivek Raja, 1980). The release of gametes, in nature is effected by the contraction of the muscular bands which is directly under the stimulatory effect of the radial nerve hormone (Cochran and Engelmann, 1972). The gamete discharge may be induced by acetylcholine, potassium chloride or by electrical stimulation.

The gamete discharge induced by electrical stimulation or by potassium chloride lasts as long as the stimulation continues. That induced by acetylcholine falls off abruptly a few minutes after injection but may be made long lasting by pretreatment with eserine. Gamete discharge induced by acetylcholine is inhibited by tubercurarine, hexamethonium and magnesium sulphate while electrically or potassium-induced discharge is not (Iwata and Fucase, 1964 a, b).

22.2 MATERIALS

Adult sea urchins (male and female).

22.3. REAGENTS

0.53 M Potassium chloride : Dissolve 39.5 gms of potassium chloride in 1000 ml of water.

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22.4. PROCEDURE

I. Direct Method

1. Select a healthy ripe sea urchin around 5 cm diameter from the rearing tank. Active movement of spines indicates the healthy condition of the specimen.
2. Measure the test diameter with the help of calipers.
3. Cut open the test with bone cutter or by scissors. Take the upper half of the animal in your hand and observe the five gonads in the inner side of aboral test.
4. With the help of forceps and small scissors remove one or two gonads.
5. Place the gonad in a Petri dish containing 20 ml of filtered sea water and tease it with the help of two fine needles. The gametes will be released into the medium.
6. Take a drop of medium along with gametes in the Pasteur pipette, place it in a slide and observe under microscope.

II. Potassium chloride method (Tyler, 1949)

1. Select a mature healthy specimen from the rearing tank.
2. Inject 0.5 ml of 0.53 M potassium chloride into the coelomic cavity through the peristomial membrane.
3. Keep the sea urchin upside down on a 100 or 250 ml beaker which is filled with filtered sea water. Make sure that the gonopores are touching the sea water.
4. Spawning begins within a few seconds after potassium chloride injection and this process continues for a period of about 15 minutes. Take a few drops of the sea water with gametes and observe it under compound microscope.

The males are generally allowed to shed into a dry dish since the sperms keep better when undiluted. Dilute the sperm just before use.

III. Electrical stimulation method (Iwata, 1962)

1. Select a healthy adult sea urchin from the rearing tank.
2. Keep the specimen on a beaker of sea water with its aboral side touching the rim of the beaker.

3. Place one of the lead electrodes on the test of the aboral side of the animal and the other on the moist cotton placed on the oral side of the animal and switch on the current supply (10-20 Volts AC).
4. Within a few seconds the animal starts spawning. Observe the released gametes under microscope.

Shedding begins shortly after the current is passed and actually ceases when it is interrupted. This makes the method advantageous for obtaining small amounts of eggs or sperms at repeated intervals from the same animal.

22.5. OBSERVATION

Observe the structure of oocytes, ova and sperms. The mature ovum measures 70-135 μm diameter with a small prominent nucleus of 7 μm in diameter, without any nucleolus, whereas the oocytes possess large germinal vesicle with a prominent nucleolus. The size of the oocyte varies from 8 to 100 μm diameter and the maximum size of germinal vesicle is 55 μm diameter (Vivek Raja, 1980). Measure the diameter of the oocyte, germinal vesicle, nucleolus and ova with the help of micrometer. Observe the jelly coat of the ova and measure its thickness.

22.6. REFERENCES

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PARTHENOGENETIC ACTIVATION AND DEVELOPMENT IN SEA URCHIN*

23.1. INTRODUCTION

Sea urchin eggs are ideal for parthenogenetic activation. Eversince Hertwig and Hertwig (1887) successfully induced the formation of fertilization membrane in *Paracentrotus lividus* by the treatment of chloroform, many workers have induced parthenogenetic activation in various sea urchin eggs both by physical and chemical stimuli (Harvey, 1956). Parthenogenetically activated eggs normally develop upto the pluteus stage ; but further development is reported to be very difficult (Ishikawa, 1975). In this experiment activation of sea urchin egg by double treatment with butyric acid and hypertonic sea water is described.

23.2. REAGENTS

1. *N/10 Butyric acid* : Add 9.24 ml butyric acid to 990.76 ml distilled water.
2. *2.5 M Sodium chloride* : Dissolve 146.1 gm of sodium chloride in 1000 ml distilled water.

23.3 PROCEDURE (*Loeb's method*, 1913)

1. Place the unfertilized eggs of sea urchin in 50 ml sea water with 2.8 ml N/10 butyric acid.
2. After 2 minutes transfer these to normal sea water.
3. After 20 minutes transfer the eggs to hypertonic sea water : i.e. a mixture of 50 ml sea water and 8 ml 2.5 M sodium chloride.

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt. Arts College, Nandanam, Madras-600 035.

(Note : A mixture of 2.5 M NaCl + KCl + CaCl₂ in the proportion in which these salts exist in sea water is still better than 2.5 M NaCl, since it is less injurious).

4. 20 minutes after immersion, transfer a few activated eggs to normal sea water for every 3 minutes upto one hour.

5. Each batch of eggs should be observed under microscope. Those eggs which have been just long enough in hypertonic solution begin to cleave.

23.4 OBSERVATION

Record the changes related to the formation of fertilization membrane and cleavage. Compare the process with that of normal fertilization.

23.5. INFERENCE

When the sea urchin eggs are activated by double treatment with butyric acid and hypertonic sea water the butyric acid induces the cortical changes leading to the formation of fertilization membrane and the treatment with hypertonic sea water favours or initiates the aster formation and other changes which are necessary for the cell division (cf. Ishikawa, 1975).

23.6. REFERENCES

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FERTILIZATION AND EARLY DEVELOPMENT IN SEA URCHIN*

24.1. INTRODUCTION

Echinoderms have served more extensively than any other group of animals for the investigation of basic problems of fertilization and early development. It was with sea urchins that Hertwig (1875) first effectively demonstrated the principal features of fertilization : the incorporation of the sperm into the egg and the fusion of sperm pronucleus with egg pronucleus. Sea urchins are exclusively marine organisms and the fertilization is external. Due to the semitransparent nature of the egg and less yolk content, the process of fertilization and early development can easily be observed in the living condition. In addition, the fertilization and development in sea urchin could be accomplished in the laboratory under relatively simple conditions.

In sea urchins, the cleavage is complete and equal, hence it is called *equal holoblastic cleavage*. During the process of cleavage the successive cleavage planes cut straight through the egg, at right angles to one another and the resultant blastomeres become symmetrically disposed around the polar axis. When the egg is viewed from either pole, the blastomeres are found to be arranged in a radially symmetrical form. This pattern of cleavage is called as *radial cleavage*.

24.1. PRINCIPLE

As soon as the sperm enters the egg, the vitelline membrane rises to become the fertilization membrane. A few minutes after sperm entry, the sperm head begins to swell, and changes into the sperm pronucleus. At the same time the middle piece breaks

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt. Arts College, Nandanam, Madras-600 035, and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

down and releases the centriole, around which egg cytoplasm forms the sperm monaster. The centriole stays close to the sperm pronucleus and lies at the center of the monaster which becomes larger as its rays elongate. When the ray tips reach the egg pronucleus, it suddenly moves toward the monaster centre and the sperm pronucleus. The two pronuclei remain in contact and unite to form a single nucleus, the synkarion. After syngamy the egg may divide synchronously ten times. During cleavage the cells become arranged in the form of a hollow sphere, the blastula. Embryos escape from the fertilization membrane after the tenth cleavage. The blastula pass through gastrulation, prism stage, pluteus stage, late pluteus stage, metamorphosis and young sea urchin to reach the adult stage (Okazaki, 1975).

24.3. MATERIALS

Adult sea urchins (males and females).

24.4. MAINTAINING ADULT SEA URCHINS IN THE LABORATORY

Sea urchins may be collected from the shore at low tide or by diving or dredging. They can easily be maintained in the laboratory. Most of them adapt well to running sea water tanks. They can also be maintained in the regular fish aquaria at room temperature. To avoid spawning in the aquaria the temperature should be kept from 15 to 20°C. Aeration is essential and up to a point, increased aeration improves the urchin's general health. When the animals are kept in the aquaria for long term maintenance they should not be over crowded. One volume of sea urchin requires 400 volume of sea water in the rearing tank. Sea urchins can be fed with large algae from the ocean, lettuce, frozen shrimp, hard boiled egg yolk and trout food (Hinegardner, 1975).

24.5. COLLECTION OF GAMETES FROM THE ANIMAL

Vide Expt. No. 22.

24.6. FERTILIZATION AND EARLY DEVELOPMENT

24.6.1. Procedure

1. When the shedding is complete the egg suspension should be filtered by a nylon mesh with opening of 100-150 μm

wide and discard the debris. The filtered eggs are allowed to settle in sea water, kept in the beaker.

2. Remove the excess sea water from the beaker and add fresh sea water. Repeat this twice to wash the eggs completely.
3. Eggs that do not settle rapidly are probably non-fertilizable. Therefore, discard them. Use separate set of pipettes for eggs and sperms.
4. Check the motility of sperm under microscope. Dilute one drop of 'dry' sperm in 10 ml of sea water and then add one or two drops of this mixture to 200 ml sea water containing fresh eggs in a finger bowl.
5. Stir the suspension of gametes. Remove the excess sperm from the finger bowl containing fertilized eggs by decanting the supernatant and replacing it with fresh sea water.
6. Transfer a few fertilized eggs on to a cavity slide for continuous observation of the formation of fertilization membrane and early development.

24.6.2. Observation

Observe the following cleavage stages: 2, 4, 8, and 16 cell stage. Time the series of development, using the chart (Table 1).

Record the cleavage planes during these stages and make sketches. Observe the size of blastomeres and the nature of their arrangement. Observe the blastula stage, hatching and ciliary movements, late blastula, early gastrula, late gastrula, prism stage, pluteus with two arm and pluteus with four arms.

24.7. FERTILIZATION AND EARLY DEVELOPMENT OF SEA URCHIN IN DIFFERENT TEMPERATURE

24.7.1. Introduction

In the room temperature, male and female pro-nuclei fuse 10-20 minutes after fertilization, and first cleavage occurs after 30 minutes (Vivek Raja, personal observation). Early sea urchin

TABLE 1. Time sequences in development of sea urchin *Salpinctes* *virgulata* at °C

Stage	Time after fertilisation in hours and minutes
Fertilization	
Fertilization membrane	
Two cell stage	
Four cell stage	
Eight cell stage	
Sixteen cell stage	
Thirty two cell stage	
Sixty four cell stage	
128 cell stage	
Early blastula stage	
Late blastula stage	
Early gastrula stage	
Late gastrula stage	
Prism stage	
Pluteus with two arms	
Pluteus with four arms	

development has a Q_{10} of about 2 for many species. That is, lowering the temperature by 10°C doubles development time (Hinegardner, 1975).

24.7.2. Procedure

Repeat the fertilization experiment at 18°C, 28°C and 38°C and record the results in Table 2.

24.7.3. Observation

Record the data on the Table 2 and find out the Q_{10} values.

$$\text{Formula for } Q_{10} = \left(\frac{K_1}{K_2} \right)^{\frac{t_1 - t_2}{10}}$$

where K_1 & K_2 are rate of activity in t_1 and t_2 respectively,
 t_1 & t_2 are temperatures.

TABLE 2

Stages	Time after fertilization at 18°C	Time after fertilization at 28°C	Time after fertilization at 38°C
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TABLE 4. Salinity (‰) Temperature (°C)

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**FERTILIZATION AND EARLY
DEVELOPMENT IN THE POLYCHAETE
*HYDROIDES LUNULIFERA****

25.1. INTRODUCTION

The eggs of the marine polychaete *Hydroides lunulifera* are small and their fertilization is external. This animal is suitable for experimental studies due to a number of features such as the ease with which the sperms and eggs could be obtained, the prolonged breeding season and the relatively simple conditions under which fertilization and development are accomplished in the laboratory conditions. During cleavage the division is complete (Holoblastic); but the pattern of cleavage is spiral. During cleavage, rotational movement, occurring around the egg axis, leads to a displacement or inclination of the mitotic spindle with respect to the symmetrically disposed radii. Hence, the spiral cleavage is the result of oblique positioning of mitotic spindles in the blastomeres.

25.2. MATERIALS

Mature *Hydroides lunulifera* (males and females).

25.3. COLLECTION AND REARING THE SPECIMEN IN AQUARIA

Hydroides lunulifera collected from the rocky shore may conveniently be maintained in the aquaria with continuous running sea water or in the conventional fish aquaria with excess sea water and continuous aeration.

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt. Arts College, Nandanam, Madras-600 035, and N. Munusamy, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

25.4. PROCEDURE

1. Remove the worm from the tube by carefully cutting the base of the tube.
2. Tease the lower part of the worm to release the gametes. In mature females, the lower part of the worm is pinkish or orange in colour due to the accumulation of the ova.
3. Observe the gametes under microscope.
4. Keep the eggs and sperms in different watch glasses.
5. Wash the eggs twice in sea water and allow them to settle in a finger bowl.
6. Add one or two drops of sperm in 25 ml of sea water containing fresh eggs in a finger bowl.
7. Stir the suspension of gametes, and with a Pasteur pipette transfer 2 ml of this suspension to a small watch glass and observe under microscope.
8. Within a few minutes, the lifting off of the fertilization membrane followed by cleavage can be observed.

25.5. OBSERVATION

Observe the cleavage stages : 2, 4, 8, 16, 32 and 64 cell stages ; blastula, early gastrula and late gastrula. Record the time taken for each developmental stage. Observe the cleavage planes, size and nature of arrangement of blastomeres during these stages.

25.6. INFERENCE

The developing eggs are sensitive to chemicals, salinity and temperature. Study the effect of temperature, salinity and chemicals on the development of *Hydroides lunulifera* (vide Expt. No. 24).

QUANTITATIVE ASSAY OF NON-SPECIFIC
ESTERASES IN THE DEVELOPING EGG OF
*EMERITA ASIATICA**

26.1. INTRODUCTION

It is a well known fact that crustacean yolk contains a considerable quantity of storage lipids to be utilized during embryogenesis. Esterases are the main hydrolytic enzymes responsible for converting complex storage lipids into easily utilizable glycerides and free fatty acids. Esterases exist as isozymes which could be characterized histochemically after separating them on polyacrylamide gel. Different species of the non-specific esterases could be further characterized using specific inhibitors. In addition, it is also possible to assay quantitatively different levels of esterase activity both in the developing ovary and developing eggs in the pleopod. This will specifically indicate the storage of esterases during vitellogenesis and their subsequent utilization during embryogenesis.

26.2. PRINCIPLE

α -naphthol liberated from α -naphthyl acetate by the enzymatic activity of the sample combines with Fast blue RR to produce a coloured compound and this is measured at 590 nm to quantify the esterase activity.

26.3. MATERIALS

Different stages of developing eggs in the pleopods of *E. asiatica*. For the classification of eggs refer Expt. No. 20.

* Prepared and verified by S. Ezhilarasi and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

26.4. REAGENTS

1. *Substrate* : Prepare 0.003 M α -naphthyl acetate by dissolving the substrate in 1 ml of acetone. Make up this 1 ml to 100 ml with double distilled water.
2. *Arresting reagent* : Mix two parts of 1 % (W/V) Fast blue RR with five parts of 1 % (W/V) sodium laryl sulphate.

26.5. PROCEDURE

26.5.1. Enzyme source

Prepare dilute aqueous egg homogenates in chilled double distilled water. Centrifuge at 2,000 g for 10 minutes. Collect the supernatant without the contamination of lipid cap.

26.5.2. Reaction mixture

Each reaction mixture consisted of 2.5 ml of substrate, 2.5 ml of (M/15) phosphate buffer from pH 6 to 9 at an interval of 0.5 and 1.0 ml of tissue homogenate. Incubate the reaction mixtures at 37°C for 30 minutes. Arrest the enzyme reaction by adding freshly prepared arresting reagent. Read all the samples and controls against the blank at 590 nm in a spectrophotometer.

26.5.3. Control I

Mix 2.5 ml of substrate with 2.5 ml of (M/15) phosphate buffer. To this mixture add 1 ml of tissue homogenate and read immediately to note initial 'zero' reading.

26.5.4. Control II (Kapin and Ahamad, 1980)

Non enzymatic hydrolysis (self hydrolysis) of acetate substrates are commonly reported. Hence to avoid the effect of non-enzymatic hydrolysis, buffered substrate without enzyme at every pH is incubated for 30 minutes and then arrested as per the other experimental tubes.

26.5.5. Estimation of Protein (Lowry *et al.*, 1951)

Protein content of the samples were determined side by side using bovine serum albumin as standard. (For procedure refer Expt. No. 12).

Express the enzyme activity as mg α -naphthol liberated/mg protein/30 minutes.

26.6. OBSERVATION

Quantify the non-specific esterase activity in different stages of egg development. Find out the optimum pH for esterase activity by plotting the values obtained in a graph.

26.7. REFERENCES

- KAPIN, M. A. AND S. AHAMAD, 1980. Esterases in larval tissues of gypsy moth, *Lymantria dispar* (L.): Optimum assay conditions, quantification and characterizations, *Insect Biochem.*, 10 : 331-337.
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DETECTION AND CHARACTERIZATION OF ESTERASE ISOZYME BY DISC GEL ELECTROPHORESIS USING INHIBITORS*

27.1. INTRODUCTION

The non-specific esterases include different types of esterases, acetyl, aryl, carboxyl and cholin esterases. These forms are identified on the basis of their differential activity towards various inorganic inhibitors (Holmes and Master, 1968 ; Dickinson and Johnson, 1978). Similarly isozymes of a type of esterase are detected by the differences in their molecular weight, as shown by their relative mobilities in disc gel electrophoresis (Ruddle and Harrington, 1976). Knowledge on the occurrence of different types of esterases and isozymes of esterases helps to pinpoint their functional role in the lipid metabolism in different tissues.

27.2. PRINCIPLE

Non-specific esterases are fractionated on polyacrylamide gel and stained by the method of Ruddle and Harrington (1976). The coupling reaction products of naphthyl substrate with non-specific esterases are stained with a diazonium salt Fast blue RR. As the dye is more stable at the neutral pH of the phosphate buffer (Kapin and Ahamad, 1980 ; Ezhilarasi, 1982), staining of esterases are carried out only in pH 7.0 of phosphate buffer.

27.3. MATERIAL

Eggs of *Emerita astatica*.

27.4. INHIBITORS

To inhibit the activity of different esterases, the following inorganic inhibitors are used in the specified concentration.

* Prepared and verified by S. Ezhilarasi and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras-Madras-600 005.

1. *p*-chloromercuric benzoic acid (p-CMB) : 10^{-1} M (0.1 M)*
2. Ethylene-diaminetetra acetic acid (EDTA) : 10^{-1} M (0.1 M)
3. Malathion (Organophosphate) : 10^{-2} M (0.01 M)
4. Silver nitrate (AgNO_3) : 10^{-2} M (0.01 M)
5. Eserine sulphate : 10^{-2} M (0.01 M)

TABLE 1. Key for identification of different species of esterases based on inhibition reaction

Chemicals	acetyl	aryl	carboxyl	cholin
P-CMB	..			
EDTA	..	— —		
Malathion	..		— —	— —
AgNO_3	.. —	— —	— —	
Eserine sulphate	..			— —

— Partial inhibition; — — Complete inhibition (Holmes and Master, 1968 ; Dickinson and Johnson, 1978).

27.5. PROCEDURE

27.5.1. Fractionation of Non-specific Esterases

Follow the same procedure as given in Expt. No. 3.

27.5.2. Staining of Esterases

Incubate the gels in 5 ml of staining mixture (Dissolve 40 mg of α -naphthyl acetate in 1 ml acetone. Add 99 ml of M/15 phosphate buffer (pH 7.0). Dissolve 70 mg of Fast blue RR in the above solution at 37°C and store in 5 : 5 : 1 ratio of methanol, water and acetic acid).

* Molar solutions are prepared by dissolving the chemical (Gram molecular weight of the chemical X desired molar) in 1 litre double distilled water.

27.5.3. Characterization of Esterases

1. Before staining for esterases incubate the gels in the inhibitor solutions for 30 minutes separately.
2. Wash in double distilled water.
3. Stain for esterases.
4. For each experiment maintain a control without treating in the inhibitor solution.
5. Visually compare the effect of inhibition by the inhibitors and tabulate the results based on Table 1. (Oxford, 1973).

27.6. OBSERVATION

Detect the different types of esterases and isozyme of esterase in the given sample.

27.7. REFERENCES

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1. Manual of Research Methods for Crustacean Biochemistry and Physiology—CMFRI Special Publication No. 7, 1981, 172 pp.
2. Manual of Research Methods for Fish and Shellfish Nutrition—CMFRI Special Publication No. 8, 1981, 125 pp.
3. Manual of Research Methods for Marine Invertebrate Reproduction—CMFRI Special Publication No. 9, 1982, 214 pp.

Back cover : SEM picture showing surface topography of *Branchinella kugunumaensis* egg.